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THE MODIFICATION OF NATURAL HYDROCARBONS BY MICROBIAL ACTION

by

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ABSTRACT

A study of the influence of microorganisms upon the physical properties of crude oil and the bacterial oil release mechanism from sedimentary materials was carried out employing fluids native to an actual reservoir in the Lloydminster "heavy oil" area and the Athabasca oil sand as well as selected organisms.

Slight oil specific gravity and oil viscosity reductions were obtained in the presence of certain <u>Clostridium</u> species and suitable fermentable nutrient media. Reduced pressure distillations showed a slight increase in the amount of intermediate fractions. Carbon dioxide and methane were the principal metabolic products. The presence of carbon dioxide could be very significant in lowering the oil viscosity at reservoir conditions of pressure and temperature. Similar effects could also be obtained under artificial conditions of waterflooding.

Microbial oxidation by single and mixed aerobic organisms of finely dispersed oil under convenient aeration conditions resulted in an increase of the oil specific gravity and viscosity. Single cultures utilized the oil as the only source of carbon while mixed populations were grown in conventional nutrient media with an oil substrate. Reduced pressure distillations indicated an increase in the amount of heavy fractions and a decrease of the light fractions as a result of hydrocarbon oxidations and/or oil components utilization. Similar results were obtained after oil treatment with enrichment cultures isolated from soils sampled near the Lloydminster refinery.

Oil release from the oil sands was obtained by naturally occuring organisms found in the sands. Microscopic and cultural characteristics

of the organisms show that the isolates are different from the *Desulfovibrio* species.

The mechanism of bacterial oil release appears to be the conveyance of bitumen particles to the nutrient medium surface by attached carbon dioxide bubbles. It is also possible that the bacterial affinity for solid surfaces promotes the removal of oil from oil-bearing sediments. The mechanism seems to be independent of the changes in surface tension of the culture medium.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPHIC PLATES

Plate	Title	Page
12	Photomicrograph (1000 X, No Filter) of the Naturally Occuring Organisms in the Athabasca Oil Sand	73

INTRODUCTION

Hydrocarbons with the exception of certain compounds of low molecular weight like methane, have generally been considered resistant to microbial attack. Since about 1950 there has been increasing evidence that microorganisms can utilize, or at least degrade, large hydrocarbons such as those found in petroleum and its products.

A dozen different genera and more than one hundred species of microorganisms have been shown to utilize hydrocarbons for the primary purpose of gaining energy for their life processes. Although the vast majority of hydrocarbon utilizing organisms can certainly use other forms of organic matter, they are characterized by their ability to subsist upon various hydrocarbons as a sole source of carbon.

It is possible that some microorganisms modify hydrocarbons more or less indirectly through the activity upon the hydrocarbons of their excretion products. When this occurs, it does not represent a true utilization of the hydrocarbons by the microorganisms, even though it does represent instances of hydrocarbon modifications (1).

There is now, and has been for some 30 years, limited research in the area of bacteriological oil recovery. In the early 1940's, ZoBell discovered that sulfate-reducing bacteria could release oil from sedimentary materials. Interest in employing microorganisms for aiding in the secondary recovery of petroleum stems actually from microbiological investigations of source sediments as part of American Petroleum Institute Research Project 43-A. As a result of a series of publications and reports from 1943 to 1952, Petroleum Microbiology was launched as a new field of endeavor due in no small measure to the lure of employing bacteria for improving secondary recovery.

It is well established that microorganisms can grow and carry out a series of metabolic activities in subsurface rock.

> "Theoretically, bacterially produced gases and acids have infinitely greater value in promoting oil recovery than gas or acid simply injected into an oil reservoir. Once active bacteria invade a reservoir and a fermentable substrate becomes available, microfoci of gas, acid and surfactant production are distributed within the formation thus exerting an internal, diffuse influence rather than as a front. This theoretical aspect of microbiological recovery of oil making it unique, has warranted experimental application in the field" (2).

The possibility of employing bacterial action in secondary recovery of petroleum is greatest for those reservoirs with the highest permeability. Despite this limitation, technically successful field experiments have been performed in Europe in sandstone reservoirs saturated with relatively high viscosity crude oil.

Primary recovery from heavy crude oil reservoirs has been very low. Sawastky (3) indicated that primary oil recovery from the Lloydminster field is only 8 per cent of the estimated original value. The recent work of Westfall (4) supports an even lower value of 5 or 6 per cent, and secondary recovery by conventional waterflooding was expected to increase 10 to 12 per cent of the initial oil in place. Low recovery by waterflooding of heavy crude oils results from reservoir stratification and unfavorable mobility ratios. Therefore, there are strong incentives to investigate the possibility of oil viscosity reductions by the action of microorganisms as a result of their excretion products and/or petroleum utilization.

In the Athabasca oil sand the hot water primary separation process produces oil as a froth which is subsequently centrifuged and refined. Currently very little is known about the mechanism of froth formation. Investigation into the mechanism of bacterial oil release

from the oil sands can contribute to an understanding of the mechanism of froth formation in the light of new data on the flotation of oil.

> "The hot water process has had the lion's share of the publicity during the past 30 years, but this is only one of several possible technologies for separating the bitumen from the sands. ...It has been estimated that up to 20 Wt % of the total deposit will be exploited by surface mining the sand and conveying it to a separation plant. The balance has too much overburden and will require in situ extraction" (5).

Therefore, any technological breakthrough will increase the number of alternatives to recover the oil from the oil sands using the in situ approach.

The purpose of this work was to investigate the effect upon the physical characteristics of heavy crude oil caused by metabolic products of selected microorganisms, as well as by biochemical oxidations produced by hydrocarbon-utilizing organisms. Naphthenic crude oil from the Lloydminster "heavy crude" area was used in this investigation. In order to simulate reservoir conditions, natural fluids without removal of the semisolid phase of tars and asphaltenes were used in this study. It was also the purpose of this investigation to determine the mechanism of oil release by naturally occuring microorganisms in the Athabasca oil sand.

It was not the intent of this investigation to study the pathways of the microbial hydrocarbon oxidations which would involve the use of infrared spectrometry techniques, mass spectrometry analysis, tracer isotopes, and several forms of chromatography developed to identify intermediates and products.

Vigorous gas producing anaerobic bacteria were tested at the beginning of this investigation. Because of partial success in lowering

the Lloydminster crude oil viscosity using these organisms, efforts to accomplish this objective were directed toward aerobic organisms which have been reported as hydrocarbon-utilizers. For a true assessment of the hydrocarbonoclastic capacities of these organisms, it was necessary to test them with crude oil as the sole source of carbon and with adequate nutrient media to observe hydrocarbon modifications.

Mixed cultures have been reported to transform hydrocarbons more readily than single cultures. An almost infinite number of organisms which are able to attack hydrocarbons can be tested. However, the time involved in this investigation, equipment and economic limitations, restricted this research to a study of the most promising combinations, their nutrient requirements, and the physical factors affecting bacterial growth.

LITERATURE REVIEW

Petroleum or its products, in contact with water, may be modified by the action of microorganisms. There is a large number of organisms that attack and transform hydrocarbons.

The statement that a microorganism is able to utilize some substrate implies that the substance concerned can be put to some valuable purpose by that particular organism (6). The reactions by which most bacteria secure energy are oxidations. Before an organism can use nutrients of high molecular weight, it must be able to break them down extracellularly into molecules small enough to pass through its cytoplasmatic membrane. Such extracellular digestion by excreted enzymes appears to play an important role in the microbial transformations of hydrocarbons by bacterial excretion products although they do not represent a true utilization of the hydrocarbons by the microorganisms.

Indirect Modification of Hydrocarbons

In 1946, ZoBell (7) suggested the use of anaerobic bacteria for injecting into petroleum reservoirs to facilitate the recovery of oil. A salt-tolerant, or salt-requiring *Desulfovibrio* species was recommended as the type of bacteria to be used in a secondary recovery process. Utilization of hydrocarbons by sulfate-reducing bacteria is an integral part of the original patent.

Updegraff and Wren (8) performed a series of experiments designed to evaluate the efficiency of oil release by sulfate-reducing bacteria. No clear evidence of petroleum decomposition by *Desulfovibrio*

bacteria was found.

A new patent by ZoBell (9) relates the recovery of hydrocarbons through the "hydrogenating" activities of Desulfovibrio, with Clostridium supplying a source of hydrogen via the fermentation of carbohydrates. The process was designed primarily for the recovery of shale oil and is for the expressed purpose of hydrogenating the oil, ostensibly making it less polar and more mobile.

Relatively little laboratory work and no field tests of bacterial secondary recovery were performed by ZoBell and his associates (10).

Updegraff and Wren (11) introduced the concept of fermentation of carbohydrates by anaerobic bacteria to produce organic acids and gas drive in the reservoir. Updegraff (12) suggested the application of this principle to a process for secondary recovery of petroleum. The bacteria recommended include those particularly adapted to fermentation of sugar, with formation of copious amounts of gases and organic acids. Those bacteria include species of Clostridium butylicum, C. butyricum, C. acetobutylicum, Bacillus macerans, B. polymyxa, and Acetobacter. In contrast with the ordinarily negative results with Desulfovibrio, oil release from sand packs and cores was readily and consistently demonstrated with sugar fermenting bacteria.

Dostalek and Spurny (13, 14) studied the microbial modification of petroleum using sulfate-reducing bacteria, genus Desulfovibrio, and denitrifying bacteria, genus Pseudomonas. An increase in specific gravity of the paraffinic oils and a preferential decrease in the paraffinic hydrocarbon fraction was reported. However, the viscosity

of the oil was decreased. When naphthenic type oil was employed the viscosity of the oil also was lowered from about 115 to 112 centistokes.

> "Carbon dioxide dissolved in the oil averaged about 0.5 ml/g and that accumulating in the atmosphere of the closed systems was about 6%. Dostalek and co-workers, concluded that viscosity lowering of oil in anaerobic systems due to bacterial action is caused by dissolved carbon dioxide. Thus a lowered viscosity occurs even though preferential utilization of the lighter molecular weight hydrocarbons by the bacteria causes an increase in specific gravity of the oil. Oils of lower specific gravity ordinarily support a greater intensity of bacterial growth. This applies particularly to naphthenic type oils.
>
> Asphaltic type oils which have a high specific gravity, and a relatively low paraffinic and naphthenic hydrocarbon content are not utilized appreciably by Desulfovibrio or Pseudomonas cultures" (15).

Field tests performed in Czechoslovakia by Dostalek and Spurny using a mixed culture of Desulfovibrio and Pseudomonas yielded a 6.85% increase in the oil recovery. No evidence was found in the field experiment of modification of the produced oil or production of appreciable quantities of gas or acids that might affect partial dissolution of reservoir rock. The bacterial production of surface active compounds was considered a possibility to explain the increase in oil recovery.

Kuznetsov (16) pointed out that growth of anaerobic bacteria in the presence of crude oils is variable and may be due to utilization of non-hydrocarbon compounds, such as: nitrogeneous, oxygenated and/or organic sulphur compounds.

In the Soviet Union, anaerobic bacteria were used for the purpose of lowering the Sernovodsk reservoir oil viscosity. Actually the viscosity of the oil was increased, but the following observations were made: pressure at the well-head was increased by 22.5 psi, oil production increased from 37 metric tons/day to 40 metric tons/day,

and the coproduced water decreased by about 25%. However, after 4 months, production dropped to 36.5 metric tons/day; water dropped another 20%. The total amount of gas production remained constant but changes in the gas composition occurred (17).

In Hungary, Jaranyi and associates (18) employed mixed cultures of anaerobic thermophillic bacteria to reduce the viscosity of naphthenic crude oil. Two tons of molasses, 100 kg of KNO_3 and 10 kg of Na_3PO_4 were used as nutrients. The original viscosity of 42.6 centistokes was reduced to 20 centistokes due to dissolved fermentation gases, after bacterial activity at 120 atm pressure. The pH of the original reservoir water was 9.0. After the bacterial treatment the pH was lowered to 7.5 due to organic acids and carbon dioxide produced from the fermented molasses. Surface tension of the coproduced water was lowered from 61.71 to 55.96 dynes/cm and gas production, particularly carbon dioxide, increased.

In Poland, field tests in sandstone reservoirs using Clostridium and other bacteria yielded oil production increases of 30 to 140 per cent with pressure increases from 2 to 25 atm in several shut-in wells which had shown a regular decrease in oil production over a period of about 20 years. In some wells production increases continued for as long as 5 years. Molasses and superphosphates were used as nutrients. The pH of the reservoir water dropped from 8.7 to 6.4. Small changes in the oil gravity and in the oil viscosity were noted. Normal distillation showed marked differences in the crude before and after the experiments. The addition of bacteria resulted in an increase in the lighter fractions and a decrease in the heavier ones (19).

In Arkansas, Yarbrough and Coty increased waterflooding oil recovery from a highly depleted sand reservoir following inoculation with a vigorous gas producing anaerobic bacterium of the genus *Clostridium*, with the addition of molasses as the fermentable nutrient source. Hydrogen, a characteristic gaseous product of sugar fermentation by the *Clostridium* was not detected in the produced gases. Due to its water insolubility it may have been trapped in the formation, thus taking up pore space and improving oil recovery by forcing floodwater to seek new channels in the formation, therefore displacing the oil (20).

Hitzman (21) suggested the inoculation of bacterial spores before the injection of the nutrients. The objective would be to distribute the bacterial spores throughout the formation. Hitzman recommended the use of *Clostridium roseum* spores, because of their small size.

Microbial Oxidation of Hydrocarbons

Biological hydrocarbon oxidation and assimilation have been discussed by ZoBell, Stewart, Fühs, Foster, McKenna and Kallio, and Van der Linden and Thijsee (22-27).

In 1961, Fühs compiled a list of organisms capable of either growing on hydrocarbons as a sole carbon source or utilizing such compounds during growth. It must be mentioned that this list was prepared from a large number of literature references and for this reason a great number of different hydrocarbons have been used as isolation substrates. About 26 genera are represented in the original list, 75 separate species have been reported as aliphatic hydrocarbon utilizers and 25 separate species as aromatic hydrocarbon utilizers.

Foster, Kester, and Lukins (28-30), performed an extensive study using saturated n-alkanes as isolation substrates. The utilization of these hydrocarbons resulted mainly in isolation of Brevibacterium, Mycobacterium, Nocardia, and Streptomyces. Other organisms such as Achromobacter, Flavobacterium, Pseudomanas, and some fungi also came up persistently as a minority. It can be seen that hydrocarbon utilization is a property distributed among bacteria, fungi and yeast. The above mentioned genera probably carry out most of the hydrocarbon degradation in nature (31).

"When microorganisms multiply in mineral salt solutions, enriched with gases, liquid or solid hydrocarbons, it is safe to assume that the hydrocarbon is utilized"(32).

In 1965, Van der Linden and Thijsse summarized the progress made through the application of new methods and techniques to the mechanism of microbial utilization of hydrocarbon oxidation. They cited numerous microorganisms related to each hydrocarbon.

Part of the original list of Fühs, as well as the organisms mentioned in the Van der Linden and Thijsse studies are summarized in the Appendix, Table A-1.

The predilection of Pseudomonas, Nocardia, and Mycobacterium could be inferred from Table A-1. At the same time, it should be noted that cycloparaffins seem to be poorly utilizable by microorganisms.

Foster (33) points out that the great majority of organisms listed by Fühs have been identified a posteriori, usually having emerged from hydrocarbon enrichment or selective cultures. The same author emphasizes the fact that the range of individual hydrocarbons which any particular organism can utilize for growth is fairly restricted.

Approaches to Oil Spills

It has been estimated that one quarter of the bacterial species on earth can attack hydrocarbons if sufficient adaptation time is provided (34).

Liu and Dutka (33) approached the oil spill cleanup problem isolating potent oil-degrading bacteria, not ordinary bacteria that readily adapt to using hydrocarbons as an energy source. The degrading bacteria were isolated from soil taken from a refinery and using the enrichment growth technique. It was found that the isolated organisms were able to degrade both the hydrocarbons and the chemical dispersants.

McLean (36, 37) reported that sump oil was "digested" by a mixed bacterial culture in Santa Barbara, California. The original oil viscosity of 1521 SSF at 180°F had a tenfold decrease after bacterial activity during one year. He stated that bacterial action is involved in the natural asphaltic material degradation whereby this material is degraded by removal of the carbon atoms to ketones, alcohols, esters, and finally to water and carbon dioxide.

There is general agreement in the fact that mixed cultures are able to transform hydrocarbons more readily and more completely than single cultures (38-41).

Bacterial Oil Release from Oil Sands

In 1947, ZoBell (42-44) reported the bacterial oil release from oil sands, after successful laboratory experiments using Desulfovibrio desulfricans species.

The role that bacteria play in decomposition of the non-hydrocarbon organic matrix of natural materials, leaving and thereby releasing the more stable hydrocarbon components, was specifically stressed by

ZoBell and associates at the Scripps Institute of Oceanography. Other bacterial mechanisms by which hydrocarbons possibly could be made more mobile were discussed. These include sulphate reduction by Desulfovibrio desulfricans, dissolution of sedimentary rock by bacterially produced hydrogen and methane, and the detergent action of bacterially produced surfactants.

> "Sulfate-reducing bacteria have at least three characteristics that dictate against their use in petroleum reservoirs for the release of oil from reservoir rock. These include their corrosive effects with respect to iron and steel, the plugging effects of colloidal iron sulfide produced in petroleum reservoirs due to generation of hydrogen sulfide by these bacteria and finally the relatively slow metabolic activity of sulfate-reducing bacteria, particularly regarding utilization of hydrocarbons" (45).

Undesirable aspects of employing sulfate reducing bacteria and consequent sulfide generation suggested the use of other anaerobic bacteria as oil releasing agents.

THEORY

General

Bacteria are simple single-cell organisms having very poorly differentiated protoplasm. They are very small, approximately 0.5 microns or less in diameter. There are three principal morphological types of bacteria: spheres which are called cocci, rods which are called bacilli, and curved rods which are called vibrio or spirilla. Some species of bacteria have motility and are able to perform a progressive movement by means of their flagella. Bacteria multiply by transverse fission. Under favorable conditions this auto-catalytic process takes place every 10 or 20 minutes. The growth or multiplication of bacteria is limited by the lack of nutrients, unfavorable environmental conditions, and by the toxic end products of bacterial metabolism. About one third of the organic matter which bacteria assimilate is converted into bacterial protoplasm, and the rest is oxidized to carbon dioxide and water or intermediate products.

Yeasts have been defined as fungi whose usual and dominant growth form is unicellular (46). Yeast cells are spherical, elliptical or cylindrical. Their sizes are highly variable and considerably bigger than the bacteria sizes. One group of yeasts reproduces by both budding and spore formation; the other reproduces only by budding.

More than a hundred species of bacteria, yeasts, and fungi are able to oxidize hydrocarbons. Others have been reported as indirect hydrocarbon transformers as a result of the action of their metabolic products upon hydrocarbons. Each species is limited in the kind of hydrocarbons it can attack. Each species is also limited by the temperature, salinity, surface tension, pH, oxygen tension, and other

environmental conditions at which it is biochemically active. Environmental conditions also affect the rate of microbial reproduction and oil oxidation (47).

Complicated chemicals may be degraded into simpler ones. What marks microorganisms apart from the rest of living systems as industrial tools is the greatly increased rate at which these transformations can take place, the fantastic range and efficiency of the chemical manipulations which microorganisms can undertake, and the relative simplicity of the technology for their application and exploitation (48).

Microbial Nutrition

Nutrients are extracellular substances that, on entering a cell after passing across the cell membrane, can be used by the cell for building material or for obtaining energy.

Practically any material on Earth can nourish one microorganism or another. An astounding list of materials can be compiled, ranging from the usual growth substances like proteins, sugars, purines, and pyrimidines to the unusual substances such as rubber, paper, leather, oil, carbon monoxide, iron, elemental sulphur, and turpentine. No organism is capable of utilizing all nutrients, and some nutrients can be used by only a small number of species.

Some microorganisms are phototrophs, able to derive their energy from sunlight. The majority, however, are chemotrophs, deriving their energy from the oxidation of chemical compounds. Chemotrophs can be divided into autotrophs, which have the ability to grow solely on inorganic materials with carbon dioxide as the carbon source, and heterotrophs, which require complex organic compounds for their main carbon source. The majority of microorganisms can transport molecules

of low molecular weight across the cell membrane but not large molecules of proteins, fats, etc. In most natural environments there are bacteria that can secrete enzymes which hydrolize larger particles to substances of lower molecular weight.

Hydrolysis is a process by which proteins, polysacharides, fats, and other large structural components are converted into their constituent parts by the introduction of water at points of cleavage of the large molecules, liberating the constituent molecules.

The selection and transport of nutrients is a function of the cell membrane. Some compounds enter the cell by diffusion, but in most instances the nutrients are transported across the cell membrane by a process called active transport. Active transport refers to the ability of an organism to accumulate substances within the cell in high concentration from an external environment in which those substances are in low concentrations (movement against concentration gradient). The transport process requires energy and is catalyzed by enzymes called permeases. Some permeases are produced only when the substrate upon which the enzyme acts is present. Thus the inability of an organism to metabolize a nutrient may reflect its inability to transport the nutrient.

Assimilation is the constructive activity by which food materials are transformed into cell constituents. Proteins, carbohydrates, fats, and other substances are produced from the simpler compounds that result from the dissimilation process. Dissimilation is the intracellular breakdown of food materials; it yields compounds that can be incorporated into new protoplasm and also the energy that makes this possible.

Bacterial Metabolism

Energy is usually transferred to a lower level. In biologic systems, however, the energy level of a compound can be raised by transferring to it certain types of chemical groups, but energy is lost or wasted in the process. In energy yielding systems, "high energy" phosphate bonds activate compounds in the building of protoplasm. The object in energy-yielding systems is to rearrange molecules to provide high-energy phosphate or other bonding.

The reactions by which most bacteria secure energy are oxidations. When a substance becomes oxidized it loses electrons; another substance receives the electrons and becomes reduced. Oxidation therefore consists of a loss of electrons accompanied by a loss of hydrogen or a gain of oxygen. Every oxidation is accompanied by an equivalent reduction of some other substance.

In the majority of biologic oxidations, hydrogen atoms are transferred from a substrate molecule, designated the hydrogen donor, and eventually passed to another substance, the hydrogen acceptor. Between the donor and the ultimate acceptor there may be several hydrogen carriers, substances that readily accept or release hydrogen.

Biologic oxidation in which molecular oxygen is the ultimate hydrogen acceptor is called aerobic respiration. The product carbon dioxide is the most highly oxidized form of carbon, and thus the greatest possible yield of energy is obtained:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + 688{,}000 \text{ cal.}$$

Chemosynthetic autotrophs secure energy by oxidizing inorganic substances: hydrogen gas, hydrogen sulfide, elemental sulphur,

iron, thiosulfate, and nitrite can also serve as autotroph's nutrient media. Some representative organisms and the proposed reactions are shown in the Appendix, Table B-1.

Anaerobic respiration is an oxidative process in which inorganic substances other than oxygen serve as the terminal hydrogen and electron acceptor. Some organisms oxidize glucose completely in the absence of atmospheric oxygen when an oxidizing agent such as potassium nitrate is present:

$$C_6H_{12}O_6 + 12KNO_3 \longrightarrow 6CO_2 + 6H_2O + 12KNO_2 + 429{,}000 \text{ cal.}$$

Sulfate reduction may yield hydrogen sulfide, and various bacteria produce methane from carbon dioxide, e.g.

$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O$$

Some typical reactions which have been proposed for anaerobic respirations of various microorganisms are shown in the Appendix, Table B-2.

Fermentation is the anaerobic decomposition of an organic substance by an enzyme system in which the final hydrogen acceptor is an organic compound. In the fermentation process, organic substances serve as both hydrogen donor and hydrogen acceptor; indeed, different parts of the same molecule often fill these roles. Since the products contain a considerable amount of energy, the yield in available energy is low:

$$C_6H_{12}O_6 \longrightarrow 2CO_2 + 2C_2H_5OH + 54{,}000 \text{ cal.}$$

In 1955, Hayaishi (48, 49) reported oxygenating enzymes or oxygenases occuring in bacteria, which were later shown to play an important role in the oxidation of aromatic and aliphatic hydrocarbons. The operation of these enzymes, which catalyze the incorporation of molecular oxygen into the substrates, in paraffin oxidation has been demonstrated in the laboratory by use of labeled gaseous oxygen (50,51). These studies, however, do not exclude the attack of the hydrocarbons in the absence of air, a phenomenon which seems to underlie the results of Azoulay et al (57) who suggested the occurence of a reversible alkane dehydrogenase in subcellular Pseudomonas particles. Conclusive demonstration of anaerobic hydrocarbon oxidation is rather difficult because of the presence of trace amounts of oxygen during the experiments and the presence of impurities which easily occur in hydrocarbon substrates.

In 1959, Leadbetter and Foster (53), during their studies on the methane-utilizing bacterium, Pseudomonas methanica, discovered the phenomenon known as co-oxidation. The organisms were unable to grow at the expense of ethane, propane or butane, but if these gases individually were present, while organisms were growing at the expense of methane, each of the co-substrates was oxidized. This technique, which they called co-oxidation was confirmed by the oxidation of alkylbenzenes by Nocardia species.

C. E. Lowery (55) has shown that co-oxidation takes place during the growth of organisms in complex mixtures of hydrocarbons.

Virtually all kinds of materials are susceptible to microbial oxidation. Degradation is the transformation of a substance into simpler components or waste. From the human viewpoint, not all breakdown

of materials by organisms is undesirable. Biodeterioration of material has been defined as any undesirable change in the properties of a material by vital activities of organisms (56).

The metabolic reactions by which aerobic microorganisms transform materials can be divided into three phases: oxidation, synthesis, and endogenous respiration. These three-phase reactions can be illustrated with the following general equations, which have been simplified from those formulated by Eston and Eskenfelder (57).

(1) Organic matter oxidation

$$C_xH_yO_z + aO_2 \longrightarrow xCO_2 + bH_2O + \text{Energy}$$

(2) Cell Material Synthesis

$$C_xH_yO_z + NH_3 + CO_2 + \text{Energy} \longrightarrow C_5H_7NO_2 + dCO_2 + eH_2O$$

(3) Cell Material Oxidation

$$C_5H_7NO_2 + 5O_2 \longrightarrow 5CO_2 + 2H_2O + NH_3 + \text{Energy}$$

In the presence of enzymes, produced by living microorganisms, about one third of the organic matter is oxidized to carbon dioxide and water to provide energy for synthesis of the other two thirds of the organic matter removed to cell material. The cell material is also auto-oxidized to carbon dioxide, water, etc., by endogenenous respiration. This mechanism suggests the presence of dissolved oxygen as in the case where the medium has been suitably aerated and agitated.

Factors Affecting the Growth of Microorganisms

Oxygen. Organisms that grow readily in the presence of air are described as aerobes. Some species are obligatory aerobes, unable to grow in the absence of free oxygen, but others are facultative anaerobes,

able to grow in its absence, though often with decreased vigour. Obligatory anaerobes cannot grow if more than a trace of free oxygen is present.

Yeast multiplication is more rapid and the yield of cells is greater under aerobic than under anaerobic conditions. Aerobic oxidation of sugar such as glucose by some yeasts can be expected to yield carbon dioxide and water plus a theoretical maximum of 688,000 calories of energy, whereas anaerobic fermentation of the same sugar to alcohol and carbon dioxide makes available no more than 54,000 calories of energy. Taking into consideration the cell energy requirements, it is obvious that aerobic conditions favor more rapid and extensive growth. Most hydrocarbon-oxidizing microorganisms require free or dissolved oxygen.

Moisture. Water is the vehicle by which microorganisms secure food and eliminate waste products. Most bacteria and yeasts prefer media of very high water content. In general yeasts require somewhat less water than most bacteria. For this reason, the knowledge of the chemical composition of the water accompanying the oil in the horizon into which the introduction of microorganisms is intended, plays an important role in the bacteriological oil recovery mechanism. A very high mineralization, the lack of phosphorous or nitrogen compounds, high acidity or high alkalinity, exert a detrimental effect on the growth of microorganisms.

Carbon Dioxide. This is probably necessary in small amounts, such as are present in the atmosphere, for the growth of microorganisms. Free carbon dioxide can be the sole carbon source for autotrophs.

Nutrient Requirements. Besides energy sources, oil oxidizers, like all other kinds of organisms, require utilizable sources of C, Ca, Mg, K, S, Fe, N, and P, plus various trace elements. A lack of utilizable compounds of nitrogen and sometimes phosphorous may limit the growth of oil oxidizing organisms. In laboratory experiments, the nitrogen and phosphorous requirements of most oil oxidizers are satisfied by the addition of several parts per million of ammonium phosphate. Equivalent quantities of other salts such as ammonium sulfate, ammonium chloride, ammonium nitrate, potassium phosphate, sodium phosphate or calcium phosphate, for example, serve the same purpose.

Temperature. The temperature of the culture medium determines the rate of growth, multiplication and death of microorganisms. The optimum growth temperature is the temperature at which most rapid multiplication occurs and is often a few degrees lower than the maximum growth temperature.

Certain by-products may be produced in greater yield or more rapidly at a temperature different than the optimum growth temperature. Temperature also affects the rates of respiration and fermentation, spore formation, pigment production, and other processes. These effects can be attributed in part to the differing temperature optima of various enzymes, in part to the greater toxicity of acids and other wastes at higher temperatures (58).

The majority of bacteria reported as hydrocarbon-utilizers are mesophiles, with original growth temperatures somewhere between 20 and 40°C. The most favorable temperature for the growth of yeasts is usually between 20° and 30°C. Incubation at 30°C is generally satisfactory.

Pressure. Bacteria seem to possess considerable resistance to mechanical or hydrostatic pressure. To date, the effects of hydrostatic pressure on organisms in the biosphere is not well understood. In all cases the pressure effect must be related to the corresponding temperature of the experiment and the amount of pressure.

> "ZoBell and Johnson studied the relationship of pressure and temperature on the growth of bacteria. Both terrestrial and marine bacteria were employed in their studies. Excellent growth of terrestrial bacteria was attained when subjected to a temperature of 20, 30, and 40°C. at 1 atm. When the hydrostatic pressure was raised to 300 atm. a noticeable decrease in the amount of growth of many terrestrial bacteria was noted at 20°C. In the majority of organisms tested, the amount of growth at 30 and 40°C. at 300 atm. was just slightly less than the atmospheric controls. At 400 atm. only a few organisms grew at 20°C. At 30°C. and 400 atm. the amount of growth was less than that occuring at 40°C. and 400 atm. None of these organisms tested were capable of growth at 20°C, and 500 or 600 atm. *Bacillus mesentericus*, *Escherichia coli* and *Streptococcus lactis* gave more growth at 40°C. at both 500 and 600 atm. than at 30°C. at 500 or 600 atm. When marine bacteria were tested under the same conditions there appeared to be a barophilic tendency of the organisms tested" (59).

Hydrogen Ion Concentration. Microbial growth and activities are strongly affected by the pH of the medium. Microorganisms differ widely in their preferences and tolerances concerning the reaction to their environment. Each species can grow only within a certain pH range, and the most rapid and luxuriant growth occurs in a narrow pH zone. Taking into consideration the fact that the nature of microbial metabolic activities is such that the pH of a culture medium does not originally remain constant after growth begins, nontoxic buffers must be added to culture media to prevent the rapid change of the pH value by acid metabolic products to a level at which organisms can no longer multiply.

Many species of yeast can multiply in solutions as acid as pH 3.0, as well as in alkaline solutions with pH 7.5. The optimum reaction is usually between pH 4.5 and 5.0.

Oxidation-Reduction Potential. The ability of an organism to grow when transferred to a fresh culture medium depends in part upon the oxidation-reduction potential. Oxidation-reduction potential is controlled by the oxidizing and reducing agents present. The various microorganisms differ with respect to the oxidation-reduction potentials at which they can begin to grow. Aerobic organisms tolerate higher potentials than anaerobes, which usually require media with negative potentials. The aeration of culture media tends to produce positive potentials, but the initiation of growth of many bacteria not only anaerobes, is favored by a somewhat lower potential.

Anaerobic Modifications of Hydrocarbons

There are few indications that microorganisms can bring about an anaerobic attack on hydrocarbon molecules. Usually hydrocarbon fermentations require much greater air input and agitation than do fermentations utilizing the more conventional carbohydrate and protein substrates. In part, this may be attributed to a problem in getting oxygen to the surfaces of the microbial cells, and in part, to the fact that considerable oxygen must be incorporated into the hydrocarbon molecule during the microbial degradation. However, metabolic products of anaerobic organisms, can contribute to modify the hydrocarbons. For instance, carbon dioxide, an end product of substrate fermentation will enter into solution at reservoir pressure and will change the physical properties of the crude oil.

In laboratory experiments, nitrate has been shown to serve as a hydrogen acceptor for certain aliphatic hydrocarbon oxidizing bacteria. Anaerobic cleavage of the aromatic ring has been demonstrated with substrates which bear a functional group, but it remains questionable whether an anaerobic utilization of aromatic hydrocarbons proper will ever be demonstrated.

Successful field tests, employing anaerobic organisms to ferment suitable nutrient media, were primarily interested in determining the possibility of a subsurface bacterial fermentation in an oil reservoir, and the effect of bacterial activity upon the physical characteristics of the oil.

Microbial Oxidation of Hydrocarbons

Under adequate nutrient media and favorable conditions, some microorganisms are known to metabolize petroleum compounds, especially hydrocarbons, via oxidative pathways, Even under optimal conditions for microbial growth, the heavy, complex hydrocarbon molecules are not rapidly metabolized to either stable cellular components or the oxidative end products.

The microbial oxidation of hydrocarbons nearly always results in the formation of water, carbon dioxide, microbial cell substance or biomass and a great variety of other end products, many of which have not been identified. More than a hundred species of bacteria, yeasts, and fungi have been found to oxidize one or more kinds of hydrocarbons. Such hydrocarbon utilizers are widely distributed in oil field soil, sump waters, and coastal areas, particularly in areas subjected to frequent pollution by petroleum or refinery products.

The microbial transformations of hydrocarbons depend upon several interrelated factors. Microorganisms capable of oxidizing hydrocarbons must be present and able to grow in the natural or artificial environment. Temperature, salinity, concentration of microbial nutrients, available oxygen, surface area of oil exposed to microbial enzymes, and the dispersion of oil in water are conditions which govern the growth and physiological activities of microorganisms.

Among the many factors which influence the biodegradability of hydrocarbons, molecular configuration seems to be the most important. In general, alkanes are attacked by more microbial species, more rapidly, and support more growth than either aromatic or naphthenic compounds. Within the alkanes, normal compounds are more susceptible to microbial oxidations than branched chain compounds (60-63).

Relatively few species oxidize methane, ethane or propane. Many more species have the ability to oxidize normal alkanes containing 10 to 18 carbon atoms than any other kind of hydrocarbons. With increasing chain length, alkanes seem to be increasingly more refractory to microbial oxidation, which may be attributable more to lower solubility in water than to lack of vulnerability to enzyme action. Branched paraffins show decreased or even negligible oxidation rates (64).

Olefins serve as substrates for a wide variety of microorganisms, although many more species exhibit a preference for saturated compounds (65).

Cycloparaffins seem to be poorly utilizable by microorganisms. Bacteria that would enable the study of cycloparaffins transformation have only been encountered twice (66).

Benzene is metabolized by a variety of microorganisms. Introducing alkyl groups into the benzene ring tends to render the resulting compound more susceptible to microbial oxidation. In general, bacteria oxidize polycyclic hydrocarbons more readily than benzene. Naphthalene is dissimilated more rapidly and by more microbial species (67).

Peroxides and hydroperoxides appear to be the primary products of the liquid phase oxidation of unsaturated, alkyl aromatic and alicyclic hydrocarbons (68).

The kinetics of the oxidation depend on the oxygen partial pressure, temperature of the environment and hydrocarbon structure. The following degradation scheme seems to take place (69).

$$\begin{tikzcd} & \text{Alcohols} \arrow[d] & \\ \text{Hydroperoxides} \arrow[ur] \arrow[dr] & & \\ & \text{Ketones} \arrow[r] & \text{Acids} \end{tikzcd}$$

Recent laboratory studies (70) showed that microbial degradation of benzene proceeds via catechol, muconic acid and 3-oxoadipic acid to give tricarboxylic acid intermediates.

Oxidation of substituted aromatic hydrocarbons presents some intriguing problems. Alkyl substituted naphthalenes, for instance, serve as microbial substrates, and are apparently attacked by either of two routes: the primary site of oxidative reaction may be the ring itself, or the presence of substitutions on the aromatic ring may force the oxidation process to the alkyl side chain (71).

Low concentration of metal salts may increase the oxidation of petroleum hydrocarbons through a free-radical mechanism. However, the mechanism is often paralyzed by the presence of oxidation-reduction

inhibitors in the hydrocarbons themselves; sulfur compounds in petroleum are sometimes potent inhibitors, though these compounds, too, may be chemically altered by microbial action.

Along with hundreds of different kinds of hydrocarbons differing in their biodegradability, most crude oils also contain small quantities of nitrogenous, oxygenated, and organic sulphur compounds. Such differences in composition help to account for great differences observed in the vulnerability of various crude oils to microbial degradation.

MATERIALS AND METHODS

Natural Reservoir Fluids

Crude Oil. Heavy crude oil from the Aberfeldy field, well C1-12-49-27W3, was used in this investigation. The Aberfeldy crude oil is a low gravity, high viscosity, black oil produced from the Sparky sand of the Mannville formation.

Oil Specific Gravity. Specific gravities of the samples at room temperature were determined using a Christian Becker specific gravity balance, model SG-1. Corrections were made to obtain specific gravity values at 60°F.

The specific gravity of the original oil used in this work was 0.9734 at 60°F. It corresponds to 13.87 degrees API gravity.

Oil Surface Tension. Surface tension measurements were made with a Cenco 70545 Du Noüy tensiometer. The corrected surface tension value of the crude oil was 31.01 dynes/cm at 22°C.

Oil Viscosity. Crude oil viscosity was measured at different temperatures and atmospheric pressure with a Brookfield rotating viscometer, model LVT. The viscometer was calibrated using calibrating oils SAE 20, 30 and 50.

Measurements of the viscosity of the crude oil with the Brookfield rotating viscometer indicated that the viscosity decreased as the length of time of agitation of the sample increased. The decrease in viscosity was considered to indicate non-Newtonian properties of the crude.

Attempts were made to determine whether the oil was thixotropic or not. To accomplish this objective, a series of measurements were made at different speeds using the #3 spindle. All the measurements were made after 5 min of agitation. The rate of shear was directly proportional to the rpm at which the measurements are made. In turn, the deflection of the needle (dial reading) was directly proportional to the shearing force. The results of these measurements are shown in Table C-1 and are plotted in Figure 1. This graph shows the thixotropic nature of the crude oil. It demonstrates that this fluid has a hysteresis effect in that the viscosity at any particular rate of shear will depend on the amount of previous shearing it has undergone. No correlation should be expected between results obtained using different spindles or when the same spindle is operated at different speeds. To overcome this problem, whenever possible, the viscosity measurements were taken with the #3 spindle, at 30 rpm, after 1 min of agitation.

It was found that the viscosity of the crude oil changes dramatically as a function of the temperature. For this reason, to determine the viscosity-temperature relationship, the thermometer was located directly in contact with the crude and not in the temperature bath.

Figure 2 shows the viscosity-temperature relationship as measured at atmospheric pressure with the #3 spindle, Brookfield LVT Viscometer at 30 rpm. All the measurements were taken after 1 min of agitation.

<u>Crude Oil Distillations</u>. Attempts at distilling the Lloydminster crude oil at atmospheric pressure, according to the Standard Method of

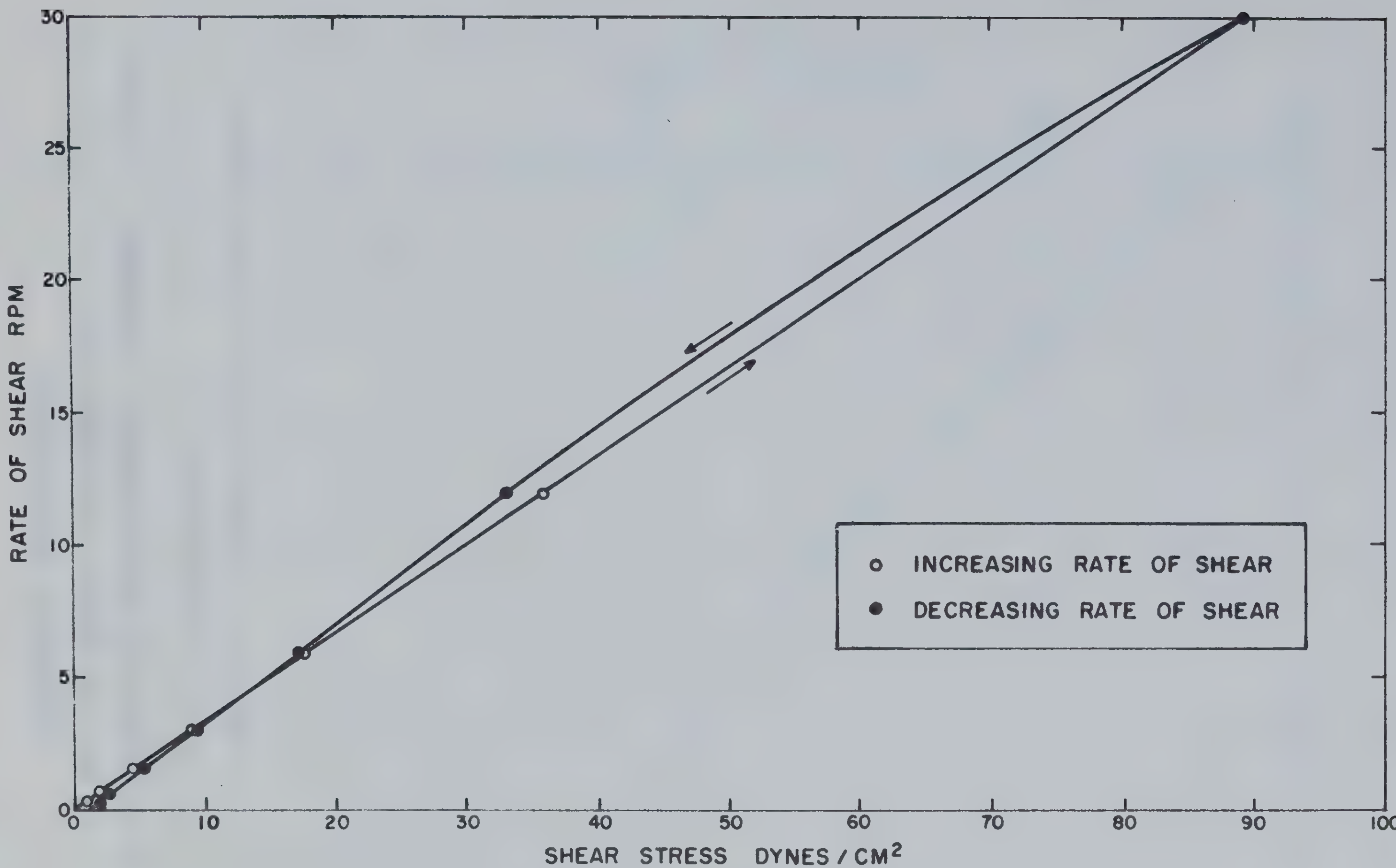

FIG. I DETERMINATION OF THE THIXOTROPIC CHARACTERISTICS OF THE ORIGINAL CRUDE OIL

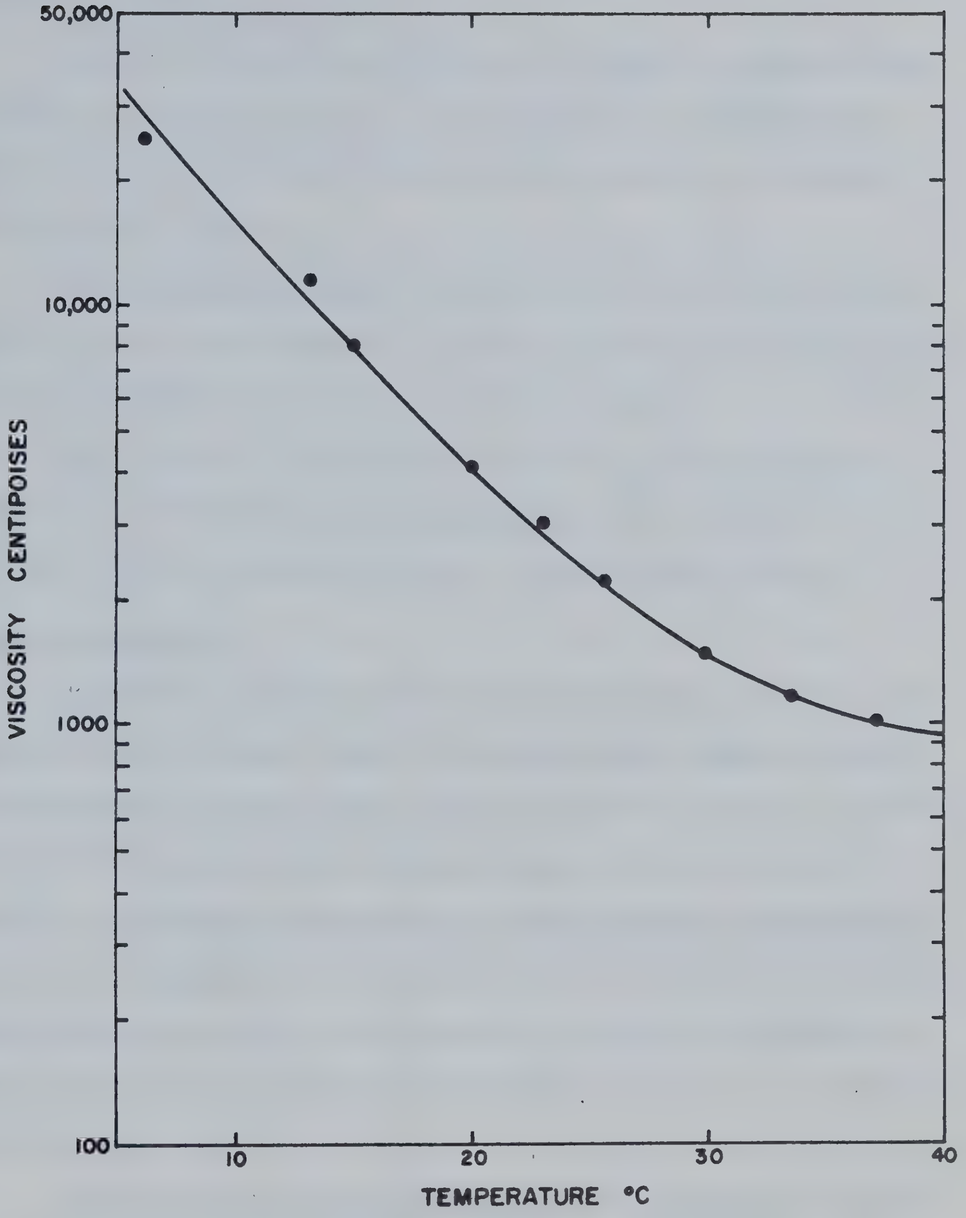

FIG. 2 VISCOSITY - TEMPERATURE RELATIONSHIP OF THE ORIGINAL CRUDE OIL AT ATMOSPHERIC PRESSURE

A.S.T.M., Designation D 285-52 (72) were unsuccessful due to cracking at the low temperature of 96.5°C.

Reduced pressure distillations of the crude oil were performed utilizing the Standard A.S.T.M. Test, Designation D 1160-52T (72). Results of the distillation of the original crude oil are presented in the Appendix, Table F-1.

Reservoir Water Analysis. Chemical analysis of the formation water is given in the Appendix, Table C-3. The very high mineralization of the Lloydminster reservoir water indicates that it is not suitable for microbial growth, even though its pH is 6.95. This conclusion is based on the observation that no *Clostridium* species growth was obtained when the formation water was used as a nutrient medium solvent.

Organisms Selection

The ability to attack and transform hydrocarbons has been demonstrated by many different types of microorganisms. These organisms include members of fungi, yeast, mycelial bacteria, bacteria, and others. This ability is particularly pronounced among various species of *Nocardia*, *Pseudomonas*, *Mycobacterium*, *Corynebacterium*, *Brevibacterium*, and the yeast *Candida*. A prime requisite for a study of microbiological hydrocarbon modifications is the presence of appropriate organisms. Consequently, careful selection of the organisms to be used in this work was necessary.

Indirect Modifications of Hydrocarbons. *Clostridium butylicum* and *acetobutylicum* had been suggested by Updegraff (74) to produce acids and gas drive in the reservoir via the fermentation of a suitable nutrient media.

Clostridium sporogenes, *C. rubrum*, and *C. septicum* were tested because of their vigorous gas producing characteristics.

Clostridium roseum spores were suggested by Hitzman (75) to distribute the small size spores (0.7 x 1.0 μm) throughout the formation.

Anaerobic thermophillic bacteria were tested in Hungary by Jaranyi (76) to reduce the viscosity of naphthenic crude oil. _Clostridium thermosaccharolyticum_ was employed in this investigation for the same purpose.

Desulfovibrio desulfricans and _Clostridium sporogenes_ were used in this work to test the "hydrogenating" activities of _Desulfovibrio_, with _Clostridium_ supplying a source of hydrogen via the fermentation of carbohydrates, as suggested by C. E. ZoBell (77).

Microbial Oxidation of Hydrocarbons. _Pseudomonas aeruginosa_ cells had been shown by various investigators (78-84) to oxidize the long chain paraffins, isoparaffins, olefins, cyclohexane, substituted aromatic hydrocarbons, naphthalene, anthracene and phenanthrene. _Pseudomonas fluorescens_ had been reported by Fühs (85) to utilize paraffin hydrocarbons. In this investigation, _Pseudomonas aeruginosa_ ATCC 17423 and _Pseudomonas fluorescens_ ATCC 17513 both isolated from hydrocarbon enrichments (86) were selected to test their oxidation abilities on the Lloydminster crude oil. _Pseudomonas rubescens_ ATCC 12099 from an oil emulsion (87) was selected with the same objective. _Pseudomonas oleovorans_ cells were reported by Lee and Chandler (88) as hydrocarbon and fatty acid oxidizers.

Gordon and Mihm (89) reported the degradation of aromatic hydrocarbons by _Mycobacterium rhodochrous_ cells.

Skopulariopsis koningii ATCC 16280, decomposes cellulose, xylane and lignin (90).

Candida lipolytica and _Candida tropicalis_ are species that are

known to utilize normal paraffinic hydrocarbons readily and unsaturated aliphatic hydrocarbons (olefins) with 12 carbon atoms or more (91).

Spores of Penicillium roqueforti are capable of converting fatty acids to ketones according to Gerih and Knight (92).

Nocardia species are highly efficient in converting even-and-odd-chain paraffins with equal facility, and producing fatty acids that are odd or even depending upon the paraffin utilized (93). According to McKenna and Kallio (94), Nocardia species are better suited to oxidize the branched hydrocarbons than are Pseudomonads and Micrococci species. The microorganisms used in studies of the transformation of ring substitute paraffins almost exclusively belong to the genus Nocardia. N. corallina ATCC 19070 and N. salmonicolor ATCC 19140 utilize alkylbenzens (95). N. corallina and N. petroleophila are known as slow hydrocarbon oxidizers.

Micrococcus cerificans ATCC 14987 produces waxes from paraffinic hydrocarbons (96). The non-volatile esters produced by the organisms do not accumulate in the cell and appear to be excreted into the culture medium (97).

Nocardia and Micrococcus species appear to play an important role in dearomatization and rupture of the benzene ring according to Van der Linden (98). Nocardia and Pseudomonas species had been shown able to degrade alkylbenzenes (99).

Experiments with Azotobacter species showed that when nitrogen fixing Azotobacter chroococcum was added to mixed cultures, less nitrogen was required for the decomposition of hydrocarbons (100).

Davies (101) indicated that _Bacillus subtilis_ and _Bacillus megaterium_ cultures could be adapted to the utilization of phenol and hydrocarbons.

Vibrio sp. ATCC 11171 isolated from sewage containing gas-works effluent is captable of breaking down aromatic ring components (102).

Techniques Employed

The methods described in this work do not constitute the only ones available. For the most part they are the methods that have been found to be the most useful to date. The many experimental difficulties that are encountered in Petroleum Microbiology present a constant challenge to the investigator and most workers in the field are constantly testing new methods.

A new microbiological technique has emerged from the work on hydrocarbon substrates. It is based on the phenomenon that a microorganism grown on a particular hydrocarbon will attack related hydrocarbons of a more complicated structure immediately, without however, oxidizing them completely.

While most studies of microbial oxidation metabolism have tested only one hydrocarbon at a time, there is strong evidence that (paradoxically) two different hydrocarbons may be more readily oxidized together than separately.

Another phenomenon noted during investigations of bacterial hydrocarbon transformation is the oxidation of a substrate followed by metabolite accumulation in the face of an apparent inability of the relevant organism to assimilate the oxidation product. The materials so oxidized have been referred to as "non-growth" substrates.

Like co-oxidation, oxidation of nonassimilable substrates may be a widespread phenomenon in the microbial kingdom.

Above all, co-oxidation and oxidation experiments mean that the inability to grow at the expense of a particular hydrocarbon is not a consequence of an organism's inability to attack the substrate. Obviously, failure to grow may be due, then, to its inability to assimilate the oxidation products. Inoculating the hydrocarbon medium with other kinds of microorganisms which can assimilate the primary oxidation products often enables the first species to grow, perhaps at the expense of intermediate products of metabolism of the mixed flora.

Most microbial species are highly selective in their ability to attack various constituents of petroleum and products formed therefrom. Mixed cultures consisting of numerous species could noticeably degrade any crude oil, but most crude oils tested have been slowly degraded.

In enrichment cultures, bacteria which utilize oil oxidation products are generally more abundant than those which attack pure hydrocarbons. In principle, the presence of abnormal amounts of hydrocarbons will elicit a growth of abnormal numbers of hydrocarbon-utilizing organisms (provided other ordinary requirements for growth are satisfied).

It was proposed in this research to study the effect upon the physical characteristics of heavy crude oil caused by metabolic products of anaerobic organisms and to investigate the hydrocarbon transformations using appropriately selected or cultivated organisms grown at the expense of diverse hydrocarbons. Mixed cultures were

also tested with the same objective. Oxidation and co-oxidation processes were expected to take place in the microbial transformation of the Lloydminster crude oil by aerobic organisms.

Nutrient Media. Whenever it was possible the organisms were cultured in various mixtures of salts dissolved in distilled water and with crude petroleum as the sole carbon source. Conventional organic nutrient media were used when the organisms were not able to grow utilizing the Lloydminster crude oil as the only source of carbon. In general, a suitable medium contained: a balanced mineral content, a nitrogen and phosphorous source such as ammonium phosphate or nitrate ions, oxygen, and a pH nearly neutral. Oxygen was excluded when testing obligated anaerobes such as *Clostridium* and *Desulfovibrio* species.

Dispersion of Oil in Water. Under optimal conditions the rate at which most liquids and solid hydrocarbons are oxidized is limited by their solubility or dispersion in aqueous media. Prerequisite to microbial oxidation, properly oriented molecules must come into contact with specific catalyzing enzymes and oxygen. This presents a problem in dispersing hydrocarbons in mineral oil solutions so that they will be available to attacking microorganisms.

Certain chemical emulsifying or oil-dispersing agents often help to make oil more available to microorganisms by increasing the contact of hydrocarbon molecules with microbial enzymes and oxygen. In experimental work in the laboratory, all due precautions must be taken to avoid adding such chemical agents because most detergents and emulsifying agents are bacteriostatic in concentrations no higher than a few parts per million and because certain oil-dispersing

agents may provide preferred sources of carbon for bacteria, thereby diminishing the oxidation of hydrocarbons.

More rapid utilization is permitted by dispersing hydrocarbons by emulsification than by adsorption on solid surfaces. In systems where the main or only source of carbon and energy is a hydrocarbon of low solubility in water, adequate hydrocarbon-water interfacial area must be provided to supply sufficient substrate to the organisms.

In this work, mechanical emulsions were used to overcome the low solubility problem and to provide sufficient hydrocarbon-water interfacial area. For this purpose, a Bellco spinner flask containing both the nutrient medium and the oil substrate was placed on a magnetic stirrer and the fluids agitated at 300 rpm. When crude oil without previous chemical treatment is stirred into the nutrient medium, a mechanical emulsion is formed. With a relatively small ratio of liquid hydrocarbon to aqueous medium, the hydrocarbon becomes dispersed as finely divided oil droplets in the aqueous phase. At high proportions of hydrocarbons to aqueous phase, however, the reverse situation is true: the phases are inverted so that finely dispersed droplets of water are suspended in the hydrocarbon. Microorganisms can grow under either situation. There is a tremendous increase in the contact area between the oil and the water (oil-water interface) as the oil is broken up into smaller droplets. For example, assuming that 200 ml of oil is broken up into spherical droplets having a radius of 0.5 mm (a very conservative assumption), a total interfacial area of 21,500 sq cm may be calculated. This area compared to the original interface of 33 sq cm indicates the magnitude of the increase in contact area that may be obtained using the Bellco Spinner flask. Since microbial growth is an interfacial

phenomenon, mechanical emulsions were employed in this work in order to accelerate the rate of hydrocarbon oxidation.

Oxygen Injection. Another problem encountered in the study of microbial oil transformations is the greater oxygen demand by hydrocarbon oxidizing bacteria. Petroleum hydrocarbons differ from those substrates that are already partially oxidized, such as carbohydrates, in that they do not contain oxygen. The need to produce the same cell essentials regardless of the carbon source requires that petroleum oxidizing organisms have a ready access to an increased supply of oxygen. This problem was approached by injecting air directly into the nutrient medium by way of the double side arm of the Bellco spinner flask.

Sterilization. A final problem in the microbial oxidation of the Lloydminster crude oil is that the more highly volatile hydrocarbons and the thick viscous hydrocarbons may be difficult to sterilize, although, in this case, it seems that sterilization is not required because of the inability of the microorganisms to utilize the hydrocarbon. One loopful of crude oil and one of reservoir water were streaked on sterile agar plates. Naturally occuring organisms were not detected either in crude oil or in the reservoir water.

Indirect Modifications of Hydrocarbons

Single cultures of the following anaerobic organisms were tested at different times: *Clostridium sporogenes* ATCC 7955, *C. septicum* ATCC 6008, *C. rubrum* ATCC 14949, *C.rubrum* ATCC 14950, *C. roseum* ATCC 17797, *C. butylicum* ATCC 14823, and *C. acetobutylicum* ATCC 824.

Thioglycollate Medium Brewer Modified BBL 11716 was used as growth medium. The nutrient medium was dispensed in a 2.0 litre pyrex bottle aspirator (outlet for tubing) in 400 ml aliquots. The

container and the medium were steam sterilized for 15 min at 121°C and 15 psi. The final pH of the medium was 7.2.

After cooling at room temperature, 200 ml of gas free crude oil were introduced into the experiment. The crude oil used in all the tests was obtained after separation of water only. The medium was aseptically inoculated with the culture of the test specie using 1.0 ml of a 24 hr growth in the same medium.

A 30 cm rubber hose with a metallic clamp was inserted into the bottom outlet of the bottle to allow for periodic culture sampling. A rubber stopper was employed to close the bottle. Two glass probes with top valves were set up through the rubber stopper for purging and gas sampling purposes.

To assure strictly anaerobic conditions, a 15 min nitrogen purge was used to remove the remaining air from the bottle. After this, the top valves of the probes were closed. The system was incubated at 37°C and the organisms were allowed to grow until the appearance of the logarithmic death phase (usually 5 days). In this case and other anaerobic tests the fluids were allowed to remain quiesent during the entire incubation period.

Culture aliquots of 30 ml were withdrawn from the fermenter every 12 hr to observe the changes in optical density, pH, and surface tension.

After 48 hours of incubation, the gas sampler was fixed to the three way cock valve and evacuated for 15 min by way of a vacuum pump. Then, the top valve was opened and the gas sample collected.

After the incubation period, both the culture and the crude oil were drained off by opening the metallic clamp of the bottom rubber hose. After the treatment, crude oil samples were collected to determine possible changes in the physical properties and chemical

composition due to the action of the bacterial metabolic products.

When the test was completed, crude oil samples from the top and the interface of the experiment were spread on Agar plates to determine the presence of living organisms in these regions. The plates were incubated at the same conditions as the original experiment. The colonies growing on the plates were aseptically smeared on microscopic slides, Gram stained, and examined microscopically for typical bacterial morphology and Gram stain characteristics.

The apparatus employed to study the anaerobic modifications of hydrocarbons is shown in Plates 1 and 2.

Molasses was investigated as a possible commercial nutrient medium. To determine the optimum concentration, solutions of 1% to 5% molasses in distilled water were prepared. Test tubes containing 5 ml of each dilution were aseptically inoculated with different *Clostridium* species. To achieve anaerobic conditions, the test tubes were placed into a GasPak anaerobic jar BBL 60645 and incubated at 37°C. The results of this investigation are shown in the Appendix, Table E-1.

A 2% molasses solution was used as growth medium for *Clostridium thermosaccharolyticum* ATCC 7956 in the presence of crude oil. The organisms were incubated at 45°C.

A fermentable substrate for the growth of *Clostridium roseum* ATCC 17797 was prepared as follows: 30 ml of molasses were added to a sterile solution of KNO_3, 0.17 g; Na_3PO_4, 1.5 g; $CaCO_3$, 0.5 g, distilled water 980 ml. The organisms were incubated at 37°C in the presence of crude oil using the same equipment and procedure described before.

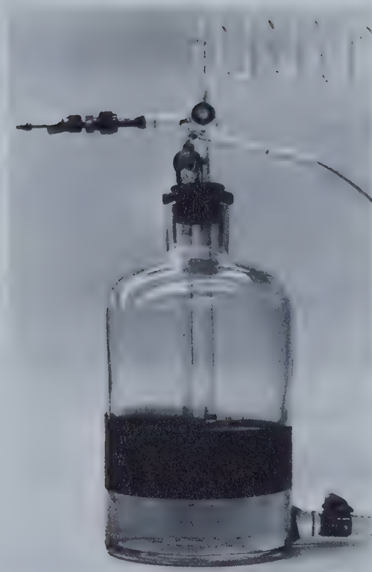

Plate 1-APPARATUS EMPLOYED TO STUDY THE ANAEROBIC MODIFICATIONS OF HYDROCARBONS

Plate 2-GAS SAMPLER EMPLOYED IN THE ANAEROBIC MODIFICATIONS OF HYDROCARBONS

A mixed culture of Desulfovibrio desulfricans ATCC 19444 and Clostridium sporogenes ATCC 7955 was tested in the presence of oil. Desulfovibrio desulfricans medium with 3% NaCl (103) consisted of: peptone 5.0 g, beef extract 3.0 g, yeast extract 0.2 g. $MgSO_4$ 1.5 g, Na_2SO_4 1.5 g, $Fe(NH_4)_2(SO_4)_2$ 0.1 g, glucose 5.0 g, and tap water 1.0 L.

The growth of some Clostridium species was studied in the presence of certain paraffinic, naphthenic, and aromatic hydrocarbons, as well as some distilled fractions of the Lloydminster crude oil. The results of this study are shown in the Appendix, Table E-2.

Treatment of the Samples

Optical Density Determination. The optical density developed from the medium by the bacterial growth was read in a Bausch and Lomb colorimeter-spectrophotometer, model Spectronic 20, at a wavelength of 600 mμ. Matched tubes were employed for the best results. To accomplish this objective, a solution of $CaCl.6H_2O$, 2.0 g and 100 ml of HCl, 0.3 M. was prepared and used as standard.

Hydrogen Ion Concentration. pH measurements were taken to an accuracy of ± 0.05 using a Beckman Glass Electrode pH Meter, model G. The pH electrodes were sterilized by washing with 500 mg/l sodium hypochloride for 10 min followed by rinsing with sterile distilled water.

Surface Tension. Surface tension measurements were made with the Du Noüy tensiometer. Surface tension of the samples were usually determined at 22°C. The measured surface tension readings were corrected using the density corrections of Zuidema and Waters (104) in graphical form. The platinum-iridium ring was sterilized by

rinsing it in chromic acid for 5 min followed by heating in the oxidizing portion of a gas flame.

Gas Chromatographic Analysis. Gas samples were quantitatively analyzed in a Beckman G-C-4 gas chromatograph. Response factors (105) were utilized to obtain quantitative results from the gas chromatographic analyses.

Microbial Oxidation of Hydrocarbons

Single Cultures. Single cultures of *Pseudomonas aeruginosa* ATCC 17423, *Pseudomonas fluorescens* ATCC 17513, *Pseudomonas rubescens* ATCC 12099, and *Pseudomonas oleovorans* ATCC 8062 were able to grow utilizing the Lloydminster crude oil as the sole carbon source in the following mineral salts distilled water solution (in grams per litre): K_2HPO_4, 0.5; $MgSO_4$, 0.5; Na_2HPO_4, 1.0; NH_4Cl, 0.5; and NaCl, 4.0. The constituents of the medium were sterilized at 121°C and 15 psi for 20 minutes. The final pH of the medium was 7.0.

The nutrient medium was dispensed in a 500 ml Bellco 3007 water jacketed spinner flask in 200 ml aliquots. In utilizing this flask, the incubator was unnecessary since the incubating temperature was supplied to the culture by a constant temperature bath and circulated through the outer jacket using a Haake R-21 thermostatic circulator. The flask was placed on a Corning PC-353 magnetic stirrer. The nutrient medium with the oil substrate was agitated at 300 rpm. Air was injected by way of the double sidearm.

The Bellco spinner flask with the nutrient medium was cooled at room temperature. Then, 200 ml of gas free crude oil was introduced into the experiment. The medium was aseptically inoculated with the culture of the test specie using 1.0 ml of a 24 hour growth

in the same medium. The system was incubated at 26°C in all the cases, except the P. aeruginosa species which was incubated at 37°C. The organisms were allowed to grow for 10 days.

Culture aliquots of 5.0 ml were withdrawn every 12 hours to observe the changes in optical density and pH. Culture and oil samples were analyzed using the equipment and techniques described in the former section. The equipment used to study the microbial oxidation of hydrocarbons is shown in Plates 3 and 4.

A test using Mycobacterium rhodochrous, which have been reported as able to grow at the expense of mineral salts solution and petrolatum as the only source of carbon (106), was performed. The organisms were grown in the original synthetic salts medium consisting of NH_4Cl, 0.5 g; $(NH_4)_2SO_4$, 0.05 g; NaCl, 0.4 g; $MgCl_2$, 0.1 g; Na_2HPO_4, 6 g; KH_2PO_4, 3 g; and distilled water 1.0 L. The medium was adjusted to pH 6.9-7.0 using a sterile 0.1 M NaOH solution and was steam sterilized. Flasks of 500 ml with wide mouths and plastic screw caps, were employed to test the Lloydminster crude oil in the presence of single aerobic organisms at a 30°C incubation temperature. The crude oil was finely dispersed using a magnetic stirrer. The bacteria cells were able to grow and to degrade the crude oil employed as the sole source of carbon.

Scopulariopsis koningii ATCC 16280 was also able to grow utilizing crude oil as the only source of carbon in the following mineral salt solution (in grams per liter of distilled water): $MgSO_4$, 0.5; Na_2HPO_4, 1.0; KH_2PO_4, 0.5; NH_4NO_3, 2.5; and $CaCO_3$, 5.0. The pH of the medium was adjusted to 7.0 and the system was incubated at 26°C.

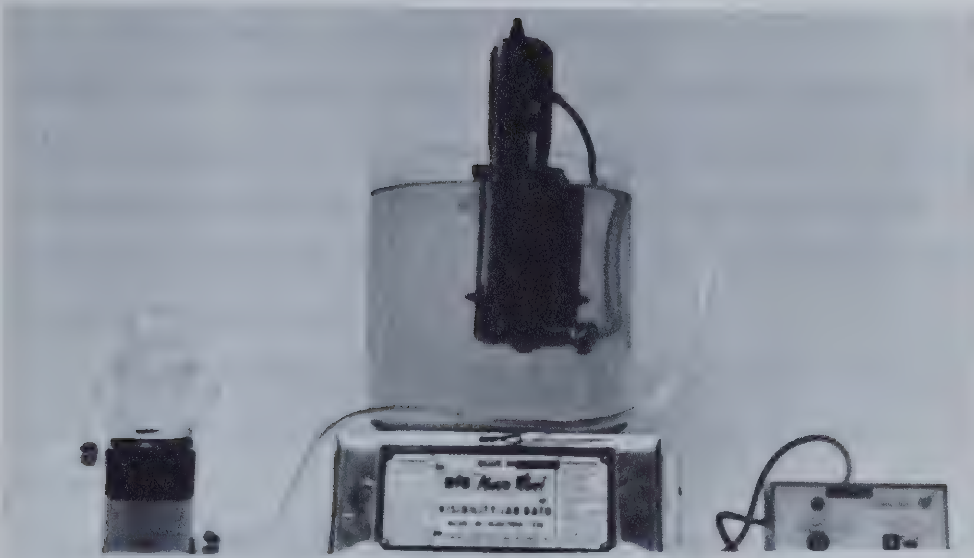

Plate 3-APPARATUS EMPLOYED TO STUDY THE MICROBIAL OXIDATION OF HYDROCARBONS: NATURAL AERATION PROCESS

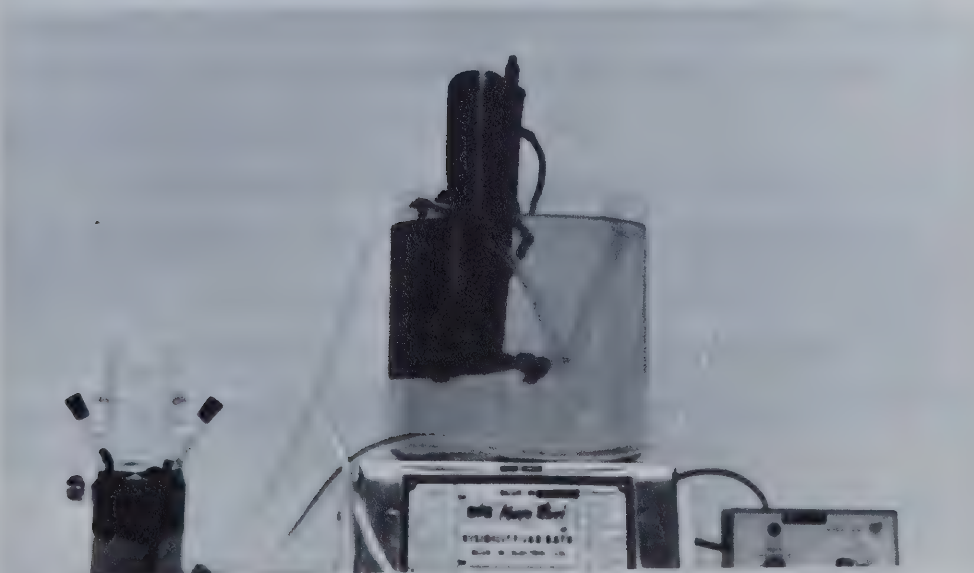

Plate 4-APPARATUS EMPLOYED TO STUDY THE MICROBIAL OXIDATION OF HYDROCARBONS: FORCED AERATION PROCESS

The following single cultures were tested in the Bellco spinner flask: Candida lipolytica ATCC 8661, Candida tropicalis (Monilia candida) ATCC 1369, and Penicillium roqueforti ATCC 6987. Tripticase soy broth BBL 11768 was used as nutrient growth medium. The final pH of the medium was 7.3. All the cultures were tested at 26°C in the presence of crude oil.

Mixed Cultures. The following mixed cultures were tested using the same equipment and procedure employed for the single cultures:

Mixed culture #1: Pseudomonas fluorescens ATCC 17513, 1 ml; Micrococcus luteus ATCC 14408, 0.5 ml; Bacillus subtilis NCIB 8640, 0.2 ml; Nocardia hydrocarbonoxydans ATCC 15104, 0.2 ml, and Azotobacter chroococcum ATCC 7493, 0.5 ml.

Mixed culture #2: Pseudomonas fluorescens ATCC 17513, 1.0 ml; Micrococcus paraffinolyticus ATCC 15582, 0.5 ml; Nocardia corallina ATCC 19070, 0.2 ml; Bacillus megaterium ATCC 14581, 0.2 ml; and Azotobacter vinelandii NCIB 8784, 0.5 ml.

Mixed culture #3: Pseudomonas oleovorans ATCC 8062, 1 ml; Nocardia salmonicolor ATCC 19149, 0.5 ml; Micrococcus cerificans ATCC 14987, 0.5 ml; and Azotobacter vinelandii NCIB 8789, 0.5 ml.

Mixed culture #4: Pseudomonas aeruginosa ATCC 17423, 1 ml; Nocardia petroleophila ATCC 15777, 0.5 ml; and Vibrio sp. ATCC 11171, 0.5 ml.

The nutrient agar Difco 001 was used as a growth medium for all the mixed cultures. The final pH of the medium was 6.9 and the incubation temperature was 26°C for mixed cultures #1, #2, and #3. A temperature of 37°C was required for mixed culture #4.

Mixed cultures #1, #2, and #4 were tested in the Bellco water jacketed flask in these two manners: agitating the liquids to promote surface absorption of air or bubbling air through the liquids. In the first case, both sidearms were plugged with non absorbent cotton. In the second one, the double sidearm was used to inject air into the system.

Crude oil samples were removed after 30 days of treatment.

Mixed culture #3 was tested using the 2.0 L bottle aspirator employed for the growth of anaerobic organisms. In this case, a nitrogen purge was not necessary and the top valves of the probes were opened, after 24 hours of growth and gas sampling, to inject air into the system.

Culture aliquots of 5 ml were withdrawn from the bottle aspirator every 12 hours during the first 5 days of growth to determine the changes in optical density and pH. The crude oil was removed from the flask after 30 days of treatment.

Enrichment Culture

The basal medium for growth of the organisms consisted of the following (in grams per liter): K_2HPO_4 anhydrous, 0.66; KH_2PO_4, 0.41; $MgCl_2.6H_2O$, 0.10; $FeCl_2.4H_2O$, 0.05; $MnCl_2.4H_2O$, 0.002, and $(NH_4)_2SO_2$, 1.0. The final pH of the medium was 6.9 and the medium was sterilized for 15 minutes at 121°C and 15 psi.

Enrichment cultures were set up using Lloydminster crude oil as the sole source of carbon. The basal medium was dispensed in a 500 ml Bellco spinner flask in 250 ml aliquots and 2 ml of crude oil was added. The medium was inoculated with 0.2 g of black soil taken from the Lloydminster refinery. The flask was placed on a magnetic

stirrer and the fluids were agitated at 300 rpm. After incubation for 3 days at room temperature, 5.0 ml of the mixed microbial cultures were transferred to fresh medium; six transfers at 4 days intervals using 2 percent inoculum and 2 percent substrate followed the initial transfer.

Pure cultures were obtained from the crude oil enrichment cultures by streaking one loopful of the enrichment culture on a sterile basal medium oil-agar plate. The carbon source was supplied by adding 0.2 ml crude oil to 15 ml liquid agar at 46°C in 2 x 15 cm test tubes. The oil was dispersed by rotating the tubes in a rotary shaker. The contents were immediately poured into precooled petri dishes. The plates were incubated in an inverted position for 3 days at room temperature (approximately 22°C). Single colonies were picked and transferred into 250 ml Erlenmeyer containing 100 ml basal medium and 2 ml oil. The flasks were incubated at room temperature on a rotary shaker operated at 300 rpm. Purity of the culture was confirmed by the repeated streaking onto oil-agar plates and gram staining.

Stock cultures were maintained on sterile oil-nutrient agar petri dishes and were transferred every 15 days; storage was at refrigerator temperature. Liquid culture for inoculum was maintained in 100 ml basal medium of which 2 percent was Lloydminster crude oil.

To test the effect of the enrichment culture upon the physical properties of the crude oil, 300 ml of basal medium and 200 ml of crude oil were dispensed in a 500 ml Bellco spinner flask and aseptically inoculated with 1 ml of the described enrichment culture. The double sidearm of the flask was plugged with non absorbent cotton. After 10 days of incubation at room temperature, the crude oil was removed to determine changes in its physical properties. Reduced pressure distillations of the oil were carried out after the treatment was

performed in order to observe any possible changes in the chemical composition.

Bacterial Release of Oil from Athabasca Oil Sand

Fifteen grams of Athabasca oil sand were placed in 2.5 x 15 cm sterile test tubes. Thioglycollate Brewer Modified BBL was used to test the probable presence of microorganisms in the oil sand. For this purpose 10 ml of Thioglycollate was added to each tube containing the oil sand sample. The tubes were tested under both aerobic and anaerobic conditions at 37°C. Anaerobic conditions were obtained in the GasPak anaerobic jar using hydrogen and carbon dioxide generator envelopes (BBL 06-112). A solution of methylene blue in Thioglycollate was employed as anaerobic indicator.

Oil release was obtained in 20 hours under both aerobic and strictly anaerobic conditions. A vigorous gas producing bacteria was observed in both cases. One loopful from each culture was streaked on a sterile agar plate and incubated under the same original conditions. Individual colonies were obtained in 20 hours. The colonies exhibited a white color. The growth was moist, glistening and abundant in appearance. Microscopic examination revealed that all the colonies were Gram-positive cocci.

To analyze the produced gas, 50 g of oil sand and 200 ml of Thioglycollate medium were incubated at 37°C in a sterile aspirator bottle with the gas sampler used to test the indirect modifications of hydrocarbons. The air was evacuated through the top valves, using a vacuum pump for 4 minutes. Gas samples were obtained after 24 hours of incubation.

Culture aliquots of 5 ml were withdrawn every 12 hours to

observe the changes in optical density and pH. In order to determine the changes in surface tension of the medium, 30 g of oil sand and 30 ml portions of the medium were placed in sterile 200 ml beakers and incubated at 37°C under both aerobic and anaerobic conditions.

Neither bacterial growth nor oil release from the oil sands were obtained at room temperature (23°C). The organisms were unsuccessfully tested at room temperature for oil release from the oil sands.

In order to test the probable presence of sulfate reducing bacteria, the following Desulfovibrio medium (107) was prepared: peptone, 5.0 g; beef extract 3.0 g; yeast extract, 0.2 g; $MgSO_4$, 1.5 g; Na_2SO_4, 1.5 g; $Fe(NH_4)_2(SO_4)_2$, 0.1 g; glucose 5.0 g; and tap water, 1.0 L. The pH was adjusted to 7.0 using a sterile 0.1M NaOH solution. With this nutrient medium, oil release from the oil sands was obtained following 20 hr of incubation at 37°C under both aerobic and anaerobic conditions.

A semi-solid Desulfovibrio medium was prepared by adding 15 g of agar to the liquid medium. One loopful of the organisms grown in Thioglyocollate and Desulfovibrio medium was streaked on a sterile Desulfovibrio medium-agar plates and incubated at the same conditions. In both cases, white colonies appeared in 20 hours. No black colonies (i.e. no sulfate reduction) were detected even after 5 days of incubation.

Sulfate reducer A.P.I. BBL 11675 medium was unsuccessfully tested for the growth of naturally occuring organisms in the oil sands. Neither growth nor oil release from the sands were obtained with this medium. The composition of the medium was the following (in grams per liter of distilled water): yeast extract, 1.0; ascorbic acid, 0.10; $MgSO_4$, 0.20; K_2HPO_4, 0.01; $Fe(NH_4)_2(SO_4)_2$, 0.10; and NaCl, 10.0. The final pH of the medium was 7.4.

An Euronbroth BBL 11235 medium was tested under similar aerobic conditions. Partial success in bacterial growth and oil release from the sands was obtained after 30 hours of incubation at 37°C.

Molasses was investigated as a possible commerical nutrient medium. Solutions ranging from 0.1 percent to 2 percent molasses in distilled water were tested. Bacterial growth and oil release were obtained in the range of 0.2 to 1.0 percent at 37°C under both aerobic and anaerobic conditions.

To determine the bacterial oil release mechanism, 15 g samples of Athabasca oil sand were placed in 2.5 x 15 cm tubes and steam sterilized for 30 minutes at 121°C and 15 psi. After cooling at room temperature, Thioglycollate and *Desulfovibrio* medium were added in 15 ml aliquots and incubated at 37°C. No growth indications were observed after 48 hours of incubation. The medium was then aseptically inoculated with the organisms isolated from the oil sands in the corresponding petri dishes. Positive bacterial growth and oil release results were obtained after 24 hours of incubation.

RESULTS AND DISCUSSION

Indirect Modifications of Hydrocarbons

The effects on the physical properties of the Lloydminster crude oil caused by metabolic products of anaerobic organisms are summarized in the Appendix, Table D-1.

Relatively slight oil viscosity reductions were obtained after the treatment with *Clostridium sporogenes* ATCC 7955. The change in specific gravity was almost negligible.

Considerable changes in the oil viscosity were obtained immediately after the treatment with *Clostridium roseum* ATCC 17797. However, measurements taken 48 hours after the treatment indicated that the original crude oil viscosity values were restored.

Figure 3 compares the viscosity of the natural crude oil with the oil viscosity after the bacterial treatment with *Clostridium sporogenes* and *Clostridium roseum*.

Clostridium roseum cultures were grown in Thioglycollate Medium Brewer Modified and in 2% molasses solution. In the second case, KNO_3, Na_3PO_4, and $CaCO_3$ were added in the proportions cited in the Materials and Methods section. The same effect for the oil viscosity was obtained in both cases, disregarding the presence of the chemicals.

The other *Clostridium* species tested in this work yielded higher oil viscosity values than the original ones. All the strains followed a consistent fermentation pattern. Evolution of fermentation gases and a decrease in pH were observed in all the cultures. Optical density determinations showed representative curves which would correspond to typical microbial population curves.

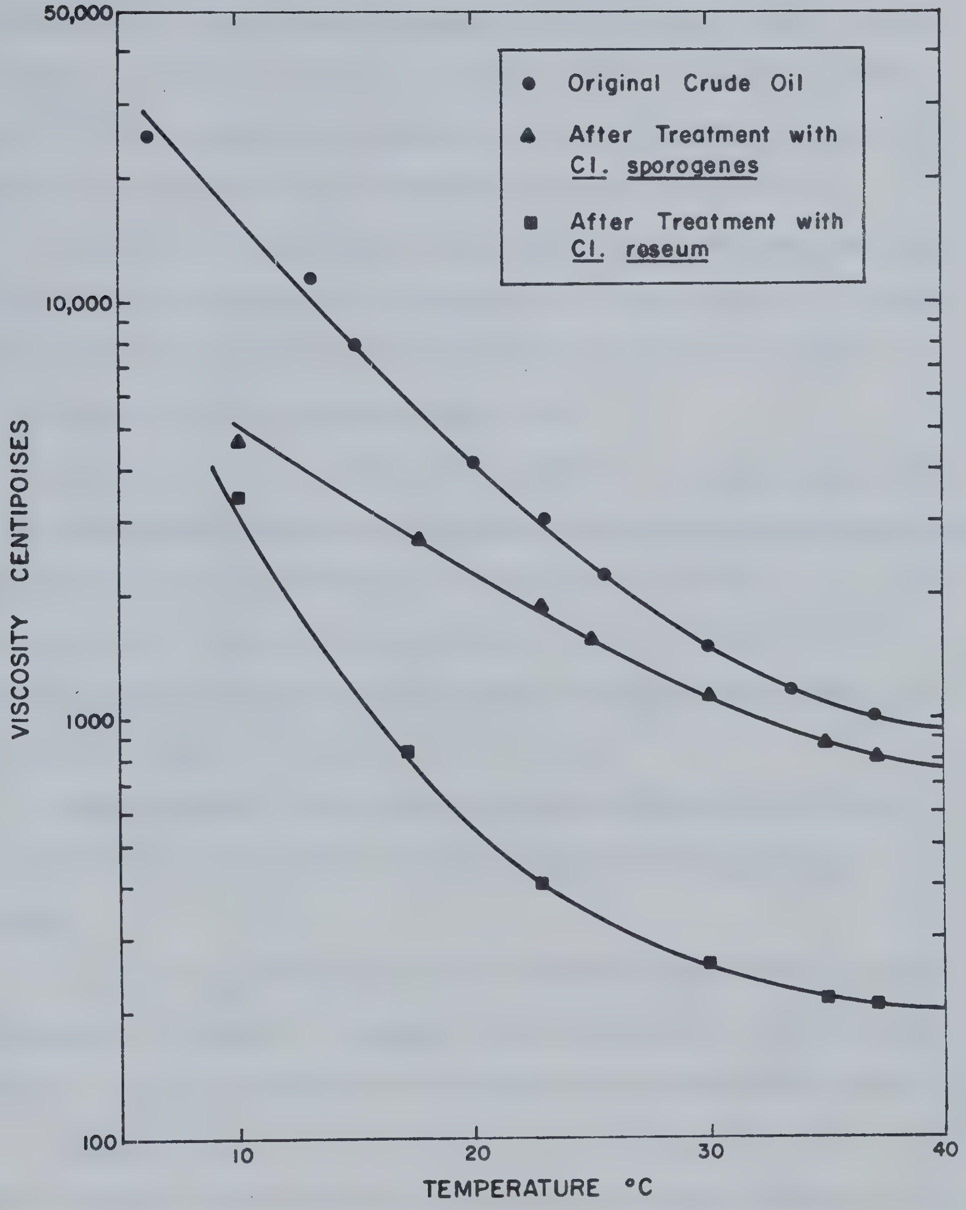

FIG. 3 VISCOSITY-TEMPERATURE RELATIONSHIP OF THE OIL AFTER BACTERIAL TREATMENT AT ATMOSPHERIC PRESSURE

Figures 4 and 5 show the pH and optical density variations corresponding to *Clostridium sporogenes* and *Clostridium roseum* cultures. In general, a decrease in pH was observed following bacterial growth. 7.10×10^8 cells per ml were determined for the *Clostridium roseum* culture using the Plate Count technique at the end of the test.

Evolution of fermentation gases through the petroleum substrate mixed the culture medium with the crude oil. Clostridium cells growing at the top of the crude oil were detected by streaking one loopful of oil on a sterile plate count agar petri dish.

Results of the gas chromotograph analysis for *Clostridium roseum* cultures with and without the oil substrate, and for *Clostridium sporogenes* cultures with the oil substrate, are presented in Tables G-1, G-2, and G-3. Results of the reduced pressure distillation of the crude oil, after the test with *Clostridium sporogenes* and *Clostridium roseum* cells are given in Tables F-2 and F-3 of the Appendix.

Reduced pressure distillations showed considerable differences in the oil composition before and after the test with *Clostridium* cultures.

At the Final Boiling Point, the recovery percent increased after the bacterial treatment. In general, the experiments in the presence of anaerobic bacteria resulted in an increase of the intermediate fractions.

Tables F-2 and F-3 of the Appendix show the results of the reduced pressure distillations of the oil following the treatment with *Clostridium sporogenes* and with *Clostridium roseum*, respectively.

As expected, large quantities of carbon dioxide, and small amounts of methane were found in the gaseous metabolic products as a result of anaerobic respirations. Laboratory experiments using

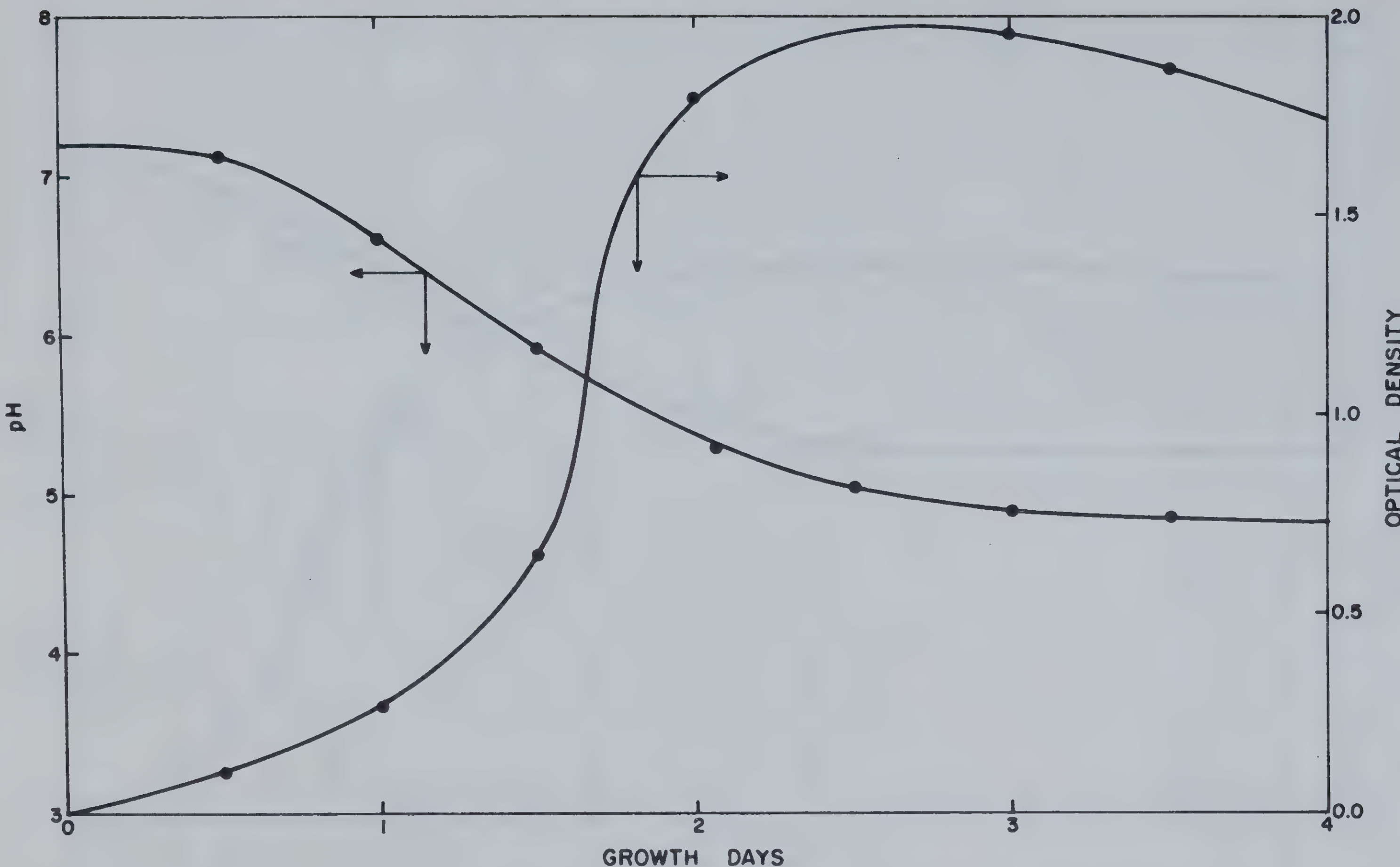

FIG. 4 CHANGE IN pH AND OPTICAL DENSITY DURING INCUBATION OF <u>CLOSTRIDIUM SPOROGENES</u> IN THIOGLYCOLLATE MEDIUM WITH CRUDE OIL SUBSTRATE

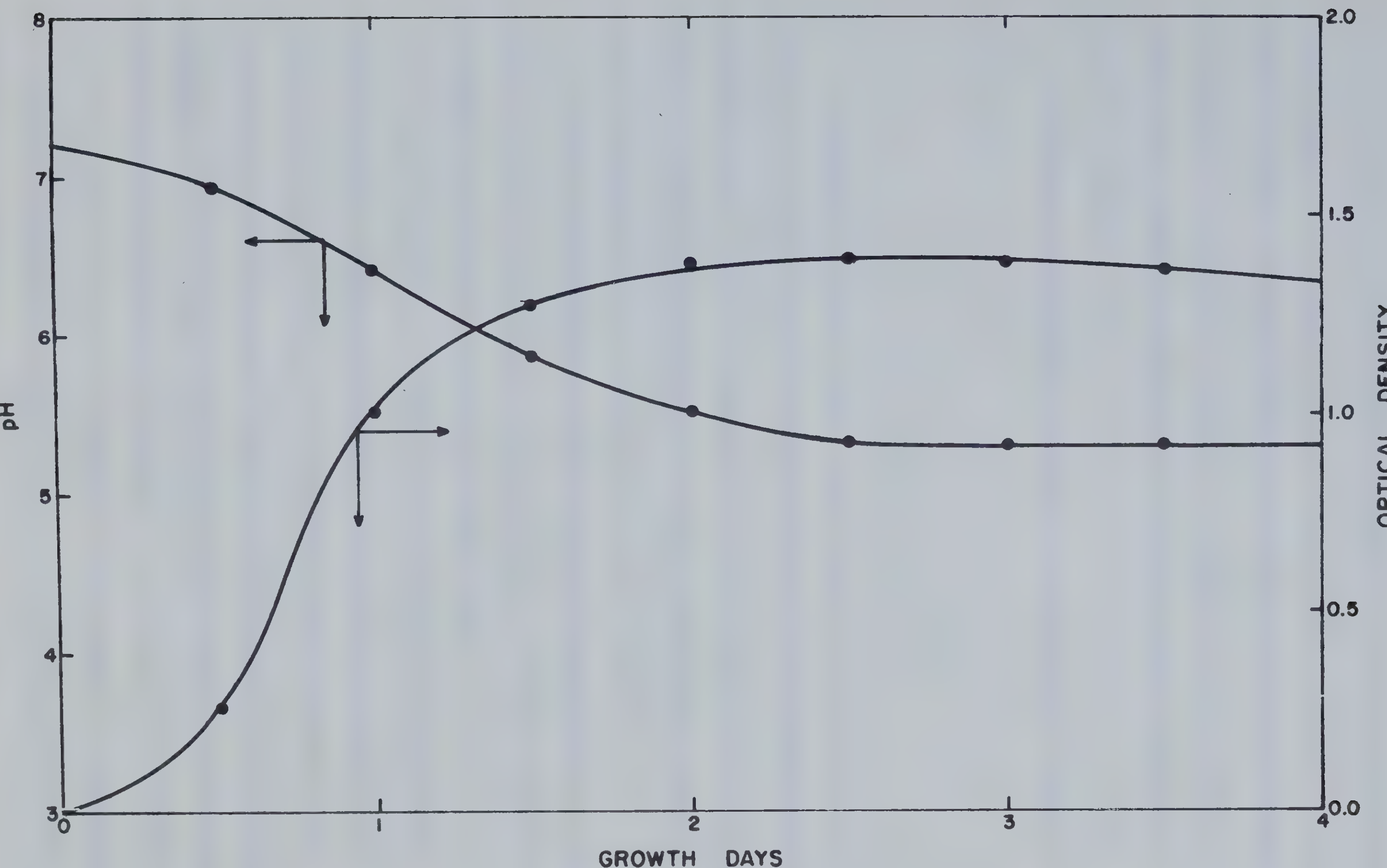

FIG. 5 CHANGE IN pH AND OPTICAL DENSITY DURING INCUBATION OF *CLOSTRIDIUM ROSEUM* IN THIOGLYCOLLATE MEDIUM WITH CRUDE OIL SUBSTRATE

Clostridium roseum cultures without a crude oil substrate showed that the principal gas produced is carbon dioxide with only 15% hydrogen. Hydrogen, however, was not produced when the organisms were tested in the presence of crude oil. Jaranyi, Yarbrough and Coty (108) have also reported the absence of hydrogen in field experiments utilizing anaerobic organisms.

Hydrogen was a probable reactant in the bacterial formation of methane, e.g.

$$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O$$

According to ZoBell (109), the microbial formation of any gaseous hydrocarbons other than methane has not been reported with conclusive proof. For this reason, the ethane production during the growth of Clostridium sporogenes and Clostridium roseum under the conditions described in this work, is rather important.

In the gaseous form at high pressure, carbon dioxide is very soluble in oil. Dissolving reasonable amounts of carbon dioxide in oil lowers its viscosity from 10-100 times (110).

Since the indirect modifications of crude oil experiments reported in this work were conducted at atmospheric pressure, only a negligible amount of gases could be dissolved in the oil. For this reason, the lowering oil viscosity effect obtained with Clostridium sporogenes and with Clostridium roseum cultures could be attributed to other excretion products, for instance, the presence of detergents or the formation of organic compounds. In any case, it represents an instance of hydrocarbon modification as revealed by the reduced pressure distillations.

The presence of carbon dioxide, methane and other metabolic gases is very significant in bacteriological oil recovery. Produced microfoci of gases are distributed within the formation thus exerting an internal, diffuse influence rather than as a front. Laboratory experiments (111) demonstrate that recovery of oil by waterflooding after gaseous fermentation of a suitable nutrient medium is consistently greater than oil recovery with plain waterflooding.

The sole presence of carbon dioxide in bacteriological oil recovery could be very important in lowering the oil viscosity at reservoir conditions of pressure and temperature. Similar effects could also be obtained under artificial conditions of waterflooding. Let us discuss the role of carbon dioxide and the presence of a free gas phase in the displacement of oil by water in porous media in the light of new data found by other investigators.

Fazil (112) found that the Lloydminster crude oil viscosity (1160 cps) was reduced to 30 cps when oil was saturated with carbon dioxide at a pressure of about 1000 psia. He also showed that carbonated waterflooding seemed to change slightly oil-wet or neutral sand towards a preferentially water-wet system.

De Nevres (113) reported that dissolving carbon dioxide in oil causes the oil to swell by about 0.35 bbl/MSCF of carbon dioxide dissolved. If residual saturation is obtained with this swollen oil, the net amount of hydrocarbon left behind will be less. In effect, some of the oil volume will be filled with carbon dioxide. In addition, carbon dioxide can react with some oils to form detergents or in some other way, to alter wetting properties of the rock-water-oil system to improve recovery.

The economic field process of oil recovery by carbon dioxide involves a carbonated water slug, followed by plain water or a slug of pure carbon dioxide followed by plain water.

Holm (114) found that neither the presence of methane (up to 15 percent volume) in carbon dioxide slugs nor a high gas saturation in the porous medium appreciably reduces the efficiency of the CO_2-carbonated water processes.

Kyte et al. (115) found that in water-wet rocks, the residual oil saturation obtained by waterflooding in the presence of a free gas phase are appreciably lower than those obtained in the absence of gas. They also suggested that in preferentially oil-wet rocks the reduction in oil saturation caused by a trapped gas depends upon the rock pore structure, the oil viscosity, and the water throughput.

Craig (116) reported that in oil-wet rocks the effect of an initial gas saturation is to reduce the injected water volume required to attain any oil recovery.

Carbon dioxide has not beem employed as the solvent agent because of the prohibitive cost to manufacture or transport it to the reservoirs where miscibility could be obtained. Taking into account that carbon dioxide, hydrogen, methane, etc., are produced as gaseous metabolic products of microorganisms and the fact that methane and hydrogen are relatively immiscible in crude oil at high pressures (i.e. 2000 psi and 150°F), bacterial gaseous fermentation of commercial nutrient media followed by formation waterflooding appears to be worthy of investigation for oil recovery from relatively homogeneous, high permeability formations, containing highly viscous crude oil.

Knowledge of the chemical composition of the water accompanying the oil in the formation in which introduction of bacteria is intended, plays an important role in microbial oil recovery. The Lloydminster formation water was analyzed and tested as a solvent medium for selected nutrient media. The chemical composition of the reservoir water is presented in the Appendix, Table C-3. It shows a high mineralization of sodium and calcium salts. Attempts to utilize the reservoir water as a solvent medium for Thioglycollate Medium Brewer Modified, failed because of the formation of insoluble precipitates.

Microbial Oxidation of Hydrocarbons

Pseudomonas species proved their ability to grow utilizing crude oil as the sole source of carbon. Similar results were obtained for *Mycobacterium rhodochrous* and *Scopulariopsis koningii* ATCC 16280.

Mycobacterium rhodochrous imparted a brown pigmentation to the crude oil and produced a very stable salt medium-oil emulsion. Viscosity measurements of the resulting emulsion showed a tremendous increase when compared with the original oil viscosity.

One of the main problems in the study of microbial modifications of hydrocarbons was to find an organism able to oxidize crude oil at a faster rate than the evaporation of the light fractions. Although some individual species of microbes are capable of attacking a variety of hydrocarbons, a mixture of microbial species will oxidize and decompose a great variety of petroleum components. Mixed saprophyte cultures have been reported (117) captable of "digesting" crude oil thereby lowering its original viscosity.

Mixed cultures of selected organisms used in this work were able to oxidize the Lloydminster crude oil at a relatively fast rate. However, the oil viscosity and the original specific gravity were increased.

Figure 6 shows the hydrogen ion concentration and the optical density variations for mixed culture #3. A slight decrease in the pH value was observed during the first 48 hours followed by a continuous increase up to a 9.5 value in 8 days. Small decreases in population were assumed from the optical density curve after the fourth day of growth. The presence of a mixed population was verified by streaking one loopful of oil on sterile plate count agar petri dishes.

Table D-2 of the Appendix presents the effect upon the physical properties of the Lloydminster crude oil caused by excretion products and/or petroleum utilization of aerobic organisms tested in this work. In general, increases in both the specific gravity and the oil viscosity were obtained after microbial activity. Table G-4 shows the results of chromatographic analysis of the gas produced by the organisms of Mixed Culture #3 in the presence of crude oil. Nitrogen and oxygen were present because of the aerobic conditions of the experiment. Therefore, the actual gas produced was carbon dioxide as a consequence of aerobic respiration by the organisms.

The increase in the oil's specific gravity could be the result of microbial utilization of the light (paraffinic) fractions and/or evaporation of the more volatile components.

Tables F-4 and F-5 of the Appendix show the results of reduced pressure distillations of the oil following treatment with *Pseudomonas aeruginosa* ATCC 17423 and mixed culture #3 respectively. The action of the bacteria resulted in a decrease of the lighter fractions and an increase in the number of heavier components. The increase in the number of heavier components seem to be due to the microbial oxidation of hydrocarbons yielding components of greater molecular weight.

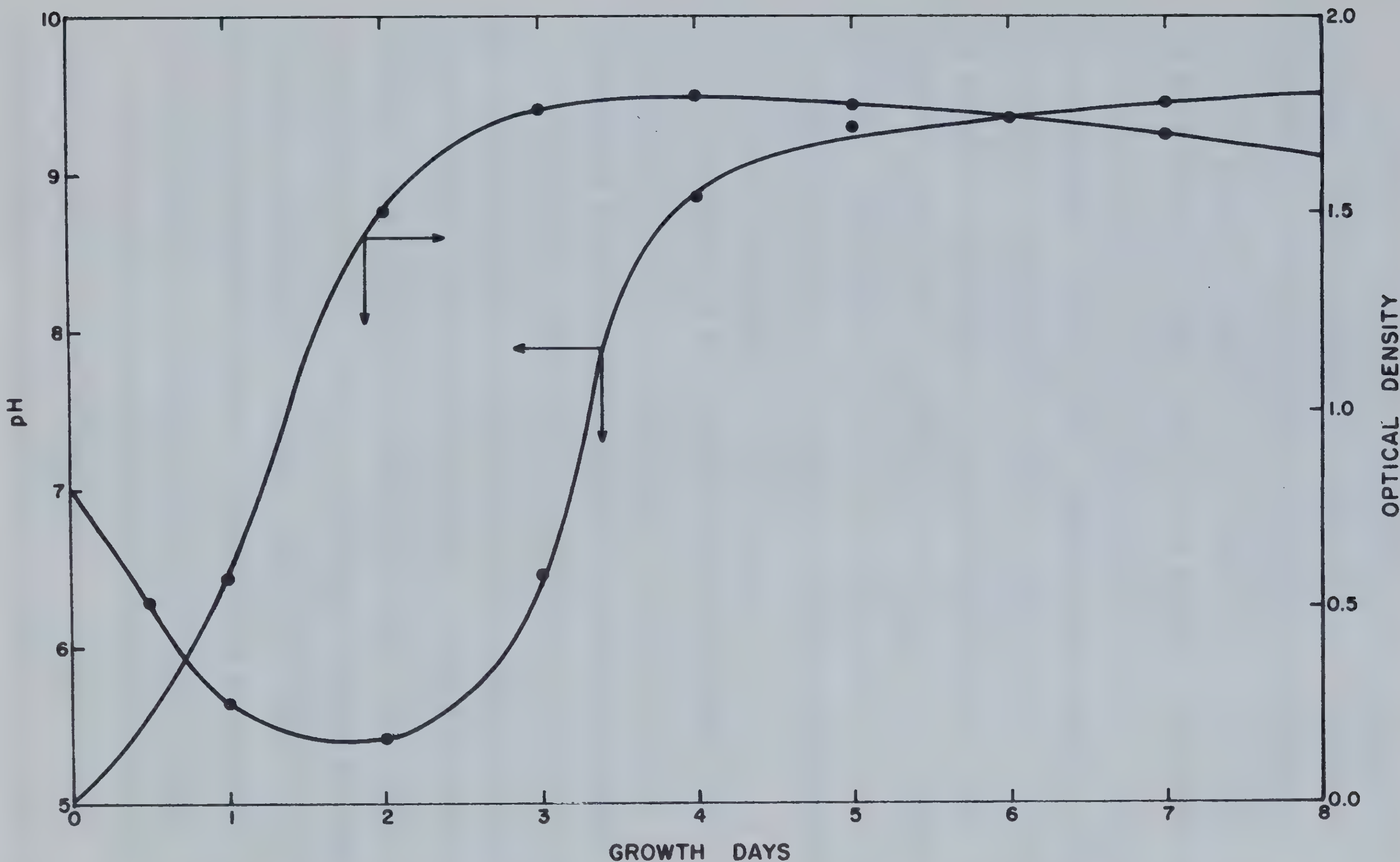

FIG. 6 CHANGE IN pH AND OPTICAL DENSITY DURING INCUBATION OF MIXED CULTURE #3 IN NUTRIENT AGAR DIFCO WITH CRUDE OIL SUBSTRATE

During the initial microbial attack process an oxygen atom is introduced into the hydrocarbon molecule. This is an important consideration because the unique characteristics of hydrocarbon oxidizing organisms appear to be associated mainly with their ability to bring about the initial attack on the molecule. Thus, many microorganisms can attack the resulting oxygenated products. The degradation of hydrocarbons after the initial oxidative attack seems to follow the general metabolic pathways common to many organisms. The effects on the physical properties of the Lloydminster crude oil after the treatment using aerobic organisms are summarized in the Appendix, Table D-2. Measurements of the oil viscosity under the same conditions of agitation and aeration as those employed during the microbial oxidation of hydrocarbons are also included.

The increase in oil viscosity after the microbial action could be explained as a result of emulsified oil, and/or oxidation of hydrocarbons yielding components with higher molecular weights.

Regardless of which type of fermentation is being considered, the initial steps of enzymatic attack on the hydrocarbon molecule usually involve an oxidation to an alcohol, followed by further oxidation to an aldehyde and finally to an acid. Thus, fatty acids of various molecular sizes often are encountered among the microbial products of hydrocarbon oxidations. Esters of alcohols and fatty acids also are commonly present. The fatty acid salts serve as emulsifying agents although they also may be further degraded.

Solid substances, insoluble in both the water and the oil phase, can also act as emulsifying agents. De Groote (118) mentioned that solid asphaltic matters or finely divided carbon can act as the common solid emulsifying agents found in crude oil.

The viscosity of oil emulsions are generally greater than the original crude oil. Emulsified oil increases the viscosity of the original crude. This is a result of the large interfacial area between the water droplets and the oil phase. The water droplets tend to act like solids thereby increasing viscosity.

Mixed microbial populations seem to be much more efficient in the oxidation of the Lloydminster crude oil than any particular microbe. Despite this, certain petroleum components are more resistant to decomposition by microorganisms than others. The situation is further complicated for the Lloydmisnter crude oil because of its naphthenic characteristics.

Thousands of different compounds with varying degrees of chemical inertness could be present in the asphaltic crude oil used in this work. In addition, some of these compounds could act as microbial inhibitors.

Enrichment Culture

A potent, oil degrading, bacteria was isolated from Lloydminster refinery soil by employing the enrichment technique. Plates 5 and 6 illustrate the development of the oil-degrading bacteria on crude-agar plates.

The isolates exhibited white colonies with occasional yellow ones. The growth was moist, glistening and abundant in appearance. Microscopic examination showed that most of the white colonies were Gram-positive cocci with occasional Gram-negative bacilli. The yellow colonies were observed Gram-negative straight and curved rods. Plates 7 and 8 show photomicrographs of the isolates.

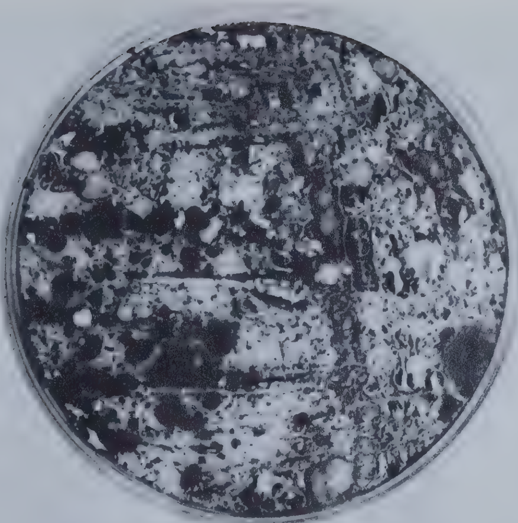

Plate 5-THE DEVELOPMENT OF OIL DEGRADING BACTERIA ON THE CRUDE-AGAR PLATE: TRANSFER #1 AFTER 3 DAYS OF INCUBATION

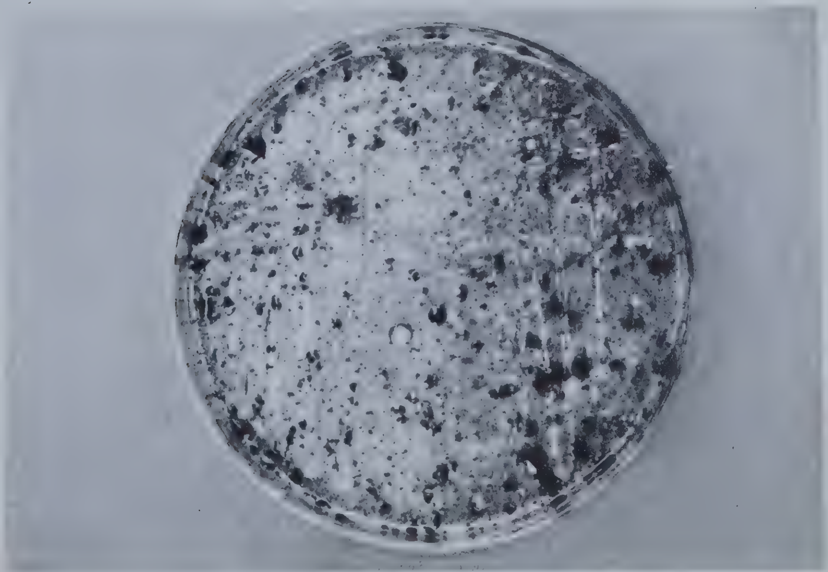

Plate 6-THE DEVELOPMENT OF OIL DEGRADING BACTERIA ON THE CRUDE-AGAR PLATE: TRANSFER #6 AFTER 3 DAYS OF INCUBATION

Plate 7-PHOTOMICROGRAPH (1000 X, FILTER) OF THE ORGANISMS CORRESPONDING TO THE WHITE COLONIES IN THE ENRICHMENT CULTURE

Plate 8-PHOTOMICROGRAPH (1000 X, FILTER) OF THE ORGANISMS CORRESPONDING TO THE YELLOW COLONIES IN THE ENRICHMENT CULTURE

All the enrichment cultures were examined for changes of pH in the medium and Gram stains were made periodically. There was a marked lowering of the pH shortly after each transfer; after remaining at approximately at pH of 6.0 for a brief period, the medium rapidly became alkaline.

No attempts were made to isolate individual colonies but rather to maintain a mixed population that quite readily oxidized the hydrocarbons and exhibited a predictable growth. One ml of the sixth transfer culture was used to inoculate the experimental vessels.

After 10 days of treatment in the presence of the enrichment culture, the Lloydminster crude oil showed the following characteristics:

	Original Crude	After Treatment
Specific Gravity, 60°F	.9734	.9870
Viscosity at 23°C, measured with the #3 spindle at 30 rpm after 60 sec of agitation	3,016 cps	6,380 cps

Table F-6 of the Appendix presents the results of reduced pressure distillation for the crude after the treatment with the enrichment culture. The presence of bacteria resulted in an increase in the heavy fractions, specific gravity and viscosity and are due to the action of microorganisms with a proven capacity to utilize the Lloydminster crude oil. There is a possibility that oxygenated compounds with higher molecular weights are formed as a consequence of the biodegration of crude oil.

Bacterial Release of Oil from Athabasca Oil Sand

Under favorable conditions of temperature and the presence of a suitable nutrient medium, oil release by naturally occuring organisms

in the Athabasca oil sand was obtained.

Using Thioglycollate Brewer Modified BBL 11716 as a nutrient medium, oil release was achieved after 20 hours of incubation at 37°C under both aerobic and anaerobic conditions. The experiments were conducted in sterile test tubes containing 15 g of Athabasca oil sand.

Plat 9 shows the bacterial oil release from the Athabasca oil sand using a Thioglycollate medium under aerobic conditions and a Desulfovibrio medium (119) under anaerobic conditions. It can be appreciated that the same amount of oil is released in both cases, regardless of which medium is used or if oxygen is present. A similar effect was obtained employing a Desulfovibrio medium under aerobic conditions. No indications of sulfate reduction were observed, even several days after the experiments.

Plate 10 compares the oil release by bacterial and steam action. Included was a control test tube, with oil sand and distilled water, which was "incubated" at the same conditions as the organisms. The naturally occuring organisms in the oil sands were grown in a 0.4% molasses solution. The third sample was subjected to steam action at 250°F and 20 psi for 30 minutes.

In all the experiments, bacterial oil release was obtained after activity of vigorous gas producing organisms. Table G-5 of the Appendix shows the results of chromatographic analysis of the gaseous metabolic products. Carbon dioxide was the main component of the gaseous mixture, resulting from respiration of the microorganisms.

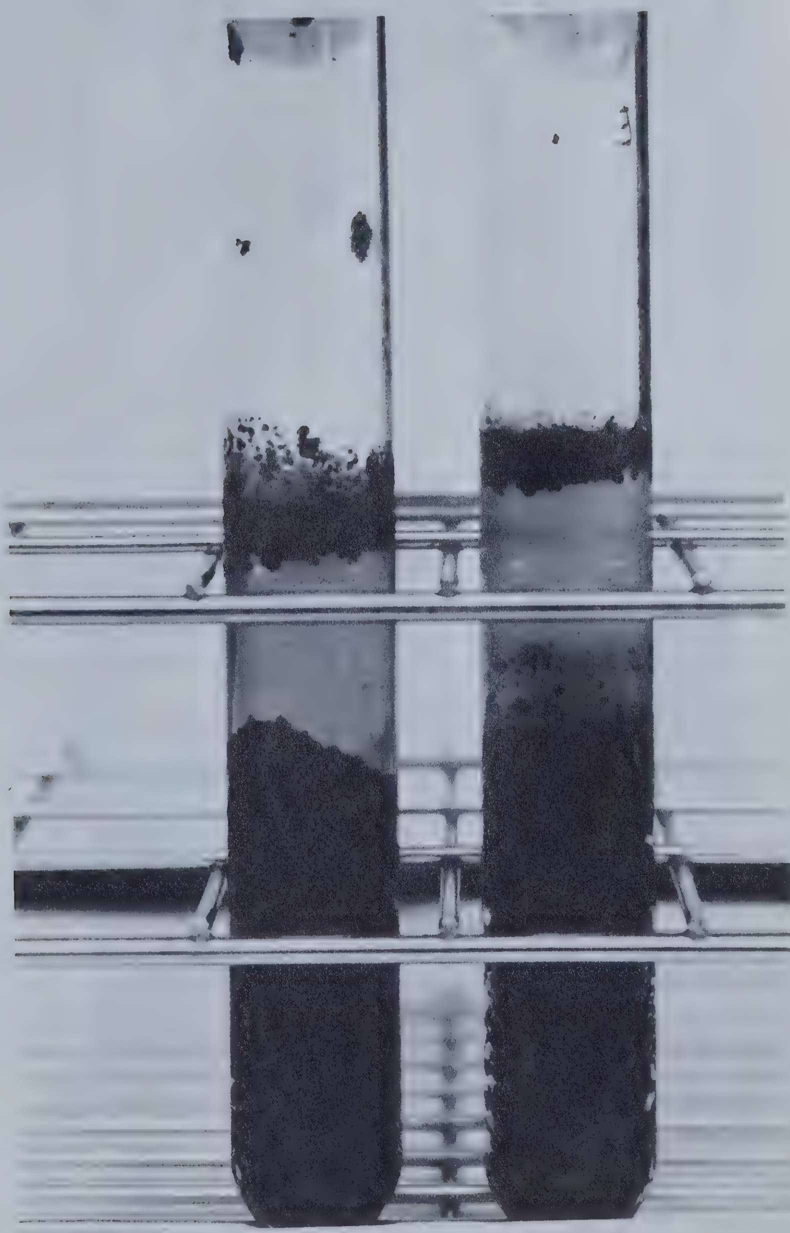

Plate 9-BACTERIAL OIL RELEASE BY NATURALLY OCCURING ORGANISMS IN THE ATHABASCA OIL SAND
LEFT: Thioglycollate Medium
RIGHT: _Desulfovibrio_ Medium

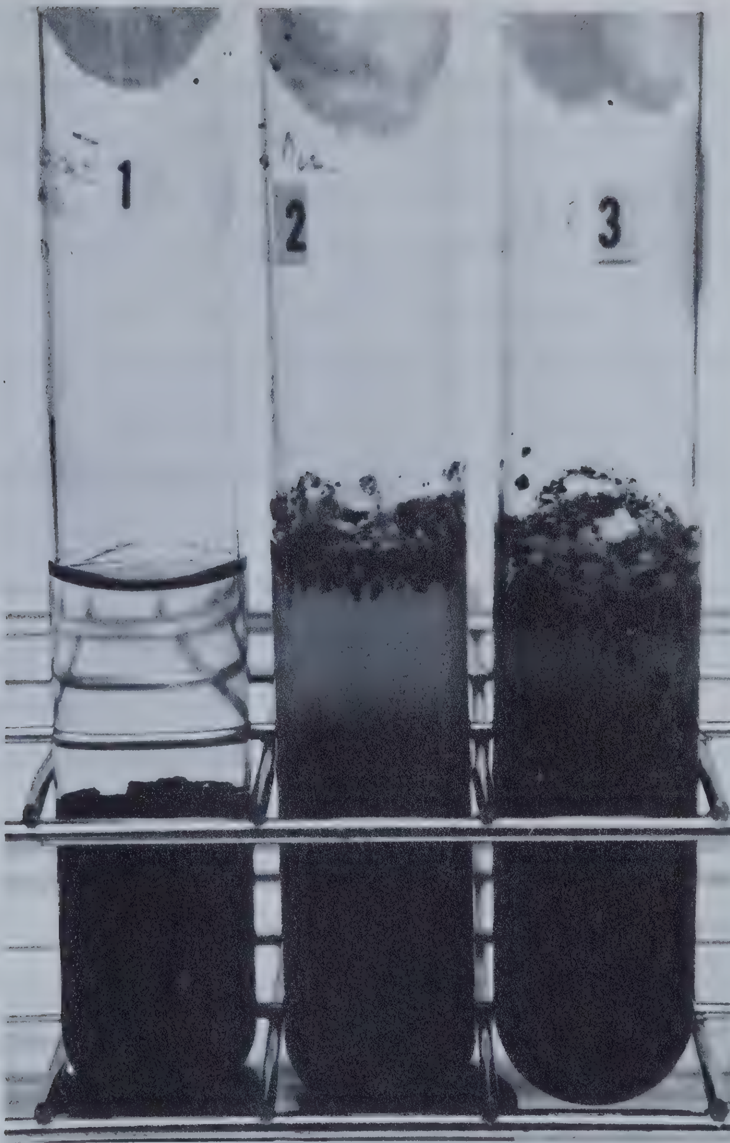

Plate 10-COMPARISON OF OIL RELEASE BY BACTERIAL AND STEAM ACTION

1. Oil sand with distilled water after 24 hrs. of "incubation" at 37°C.
2. Oil sand and distilled water after steam action at 250°F and 20 psi during 30 minutes.
3. Oil sand with 0.4% molasses solution after 24 hrs. of incubation at 37°C.

White, circular and convex colonies were observed after 24 hours of incubation in Plate Count Agar petri-dishes and Desulfovibrio medium-Agar plates. In the second case, evidence of sulfate reduction was not observed. All the colonies exhibited a characteristic white color. The growth was moist, glistening and abundant in appearance.

Plate 11 shows the colonies of the naturally occuring organisms in the Athabasca oil sand. The organisms were grown under anaerobic conditions on a Desulfovibrio medium-Agar plates.

Microscopic examination revealed that all the colonies were Gram-positive cocci in clusters (staphyloccus). Organism's motility was determined by the hanging-drop method.

Plate 12 shows a photomicrograph of the natural organisms in the Athabasca oil sand.

Molasses was investigated as a possible nutrient medium. Optimal concentrations of this nutrient are in the range of 0.2-1.0%.

Oil release was obtained in the presence of a Eugonbroth Vera BBL 11234 medium and a Trypticase Soy Broth BBL 11767. However, no bacterial oil release was achieved using Sulfate Reducer API Broth BBL 11677 medium or a synthetic salts medium. These results seem to indicate that naturally occuring organisms releasing oil from the oil sands are other than the Desulfovibrio desulfricans species.

Since bacterial oil release occurs under both aerobic and anaerobic conditions, the organisms could be described as facultative anaerobes. This finding, in addition to motility and no sulfate reduction (i.e., production of H_2S) which are characteristics of the organisms, could be very important in possible field applications.

Plate 11-COLONIES OF NATURALLY OCCURING ORGANISMS IN THE ATHABASCA OIL SAND GROWING UNDER ANAEROBIC CONDITIONS ON A DESULFOVIBRIO MEDIUM-AGAR PLATE AFTER 24 HOURS OF INCUBATION

Plate 12-PHOTOMICROGRAPH (1000 X, NO FILTER) OF THE NATURALLY OCCURING ORGANISMS IN THE ATHABASCA OIL SAND

Steam sterilized (250°F and 20 psi for 30 minutes) oil samples did not release oil after incubation at 37°C in the presence of a suitable nutrient medium. Oil release was obtained by inoculating the medium with the organisms isolated from the sands and could be obtained from any gas producing organisms, such as the *Clostridium* species.

Attempts at obtaining bacterial oil release from the sands at room temperature were unsuccessful. Consequently, the majority of the experiments were conducted at 37°C.

Figures 7 and 8 show the changes in pH and optical density of the isolates in the presence of Thioglycollate and *Desulfovibrio* media, respectively. A decrease in pH was observed in all the experiments.

Figure 9 presents the variations in surface tension of Thioglycollate and *Desulfovibrio* media. The measurements were taken at 22°C. A slight increase in the surface tension of the Thioglycollate medium was observed during the first 24 hours of bacterial growth and oil release. After this, the surface tension values started to decrease. A different effect was observed in the *Desulfovibrio* medium. During the first 24 hours the surface tension of the medium was considerably lower than the original value. However, the surface tension values started to increase in the next 48 hours. After this period, new decreases in the surface tension values were observed.

The variations in surface tension of the culture medium could be due to the production of acids, detergents or wetting agents made by the organisms.

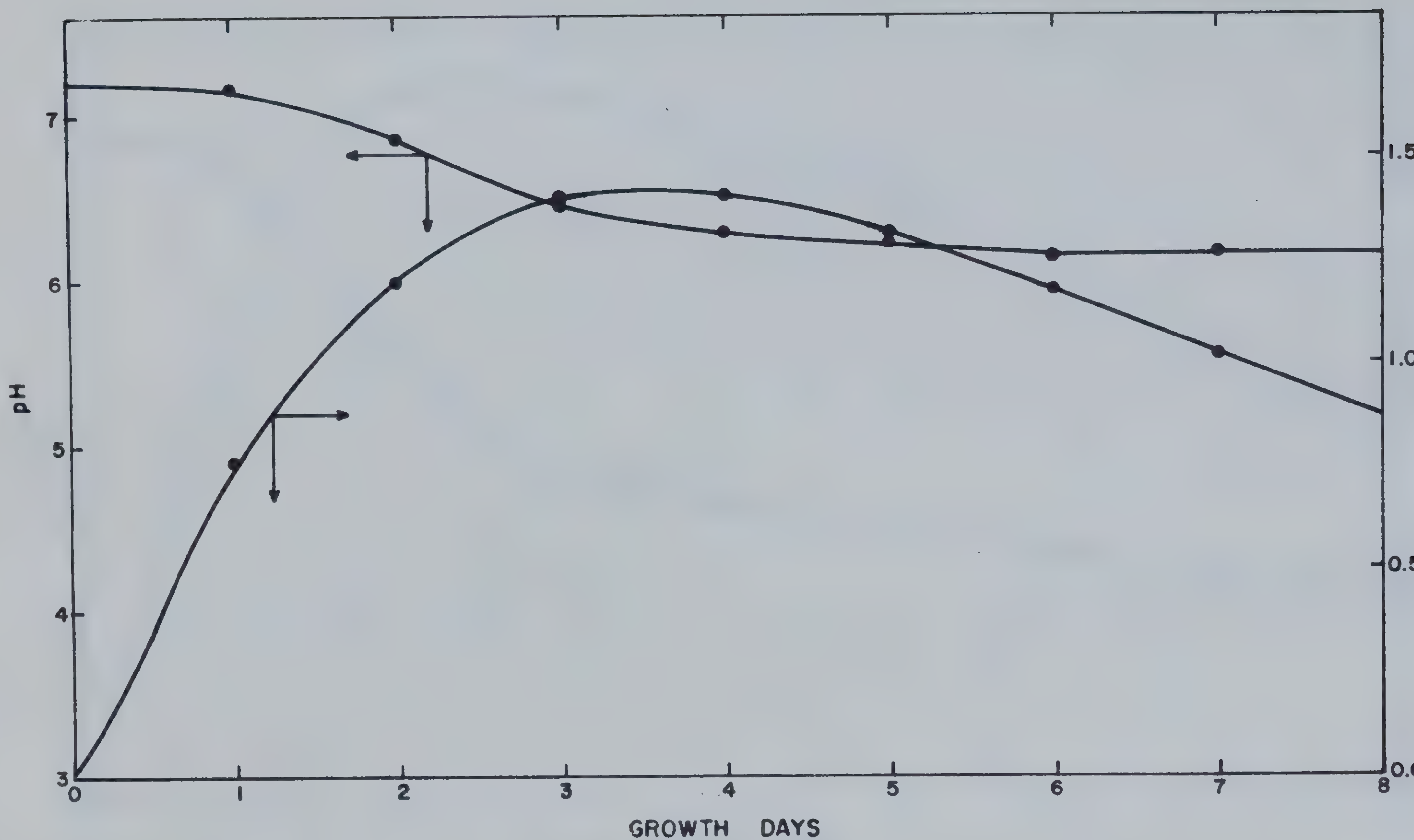

FIG. 7 CHANGE IN pH AND OPTICAL DENSITY DURING INCUBATION IN THIOGLYCOLLATE MEDIUM OF THE NATURALLY OCCURING ORGANISMS IN THE ATHABASCA OIL SAND

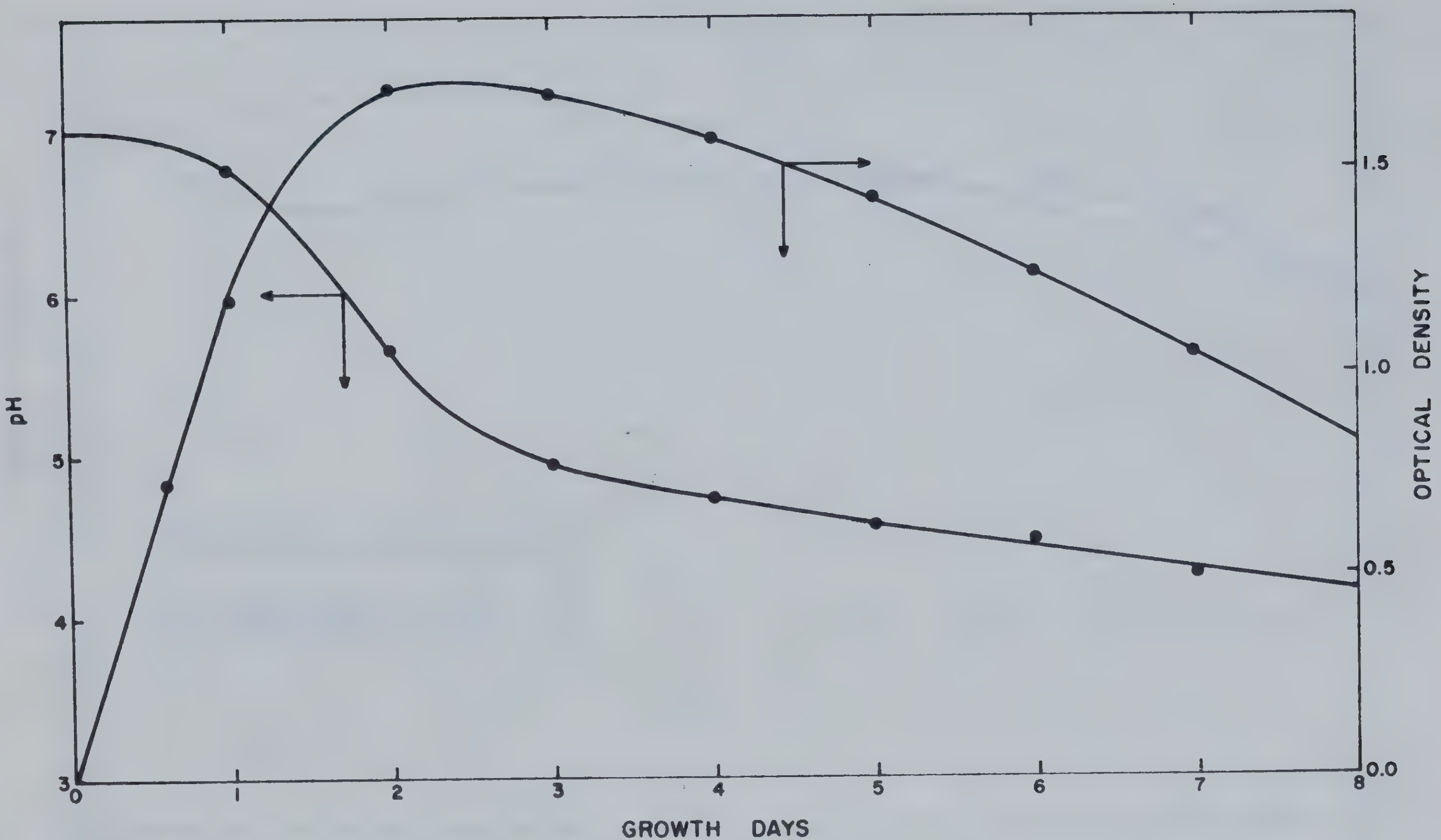

FIG. 8 CHANGE IN pH AND OPTICAL DENSITY DURING INCUBATION IN <u>DESULFOVIBRIO</u> MEDIUM OF THE NATURALLY OCCURING ORGANISMS IN THE ATHABASCA OIL SAND

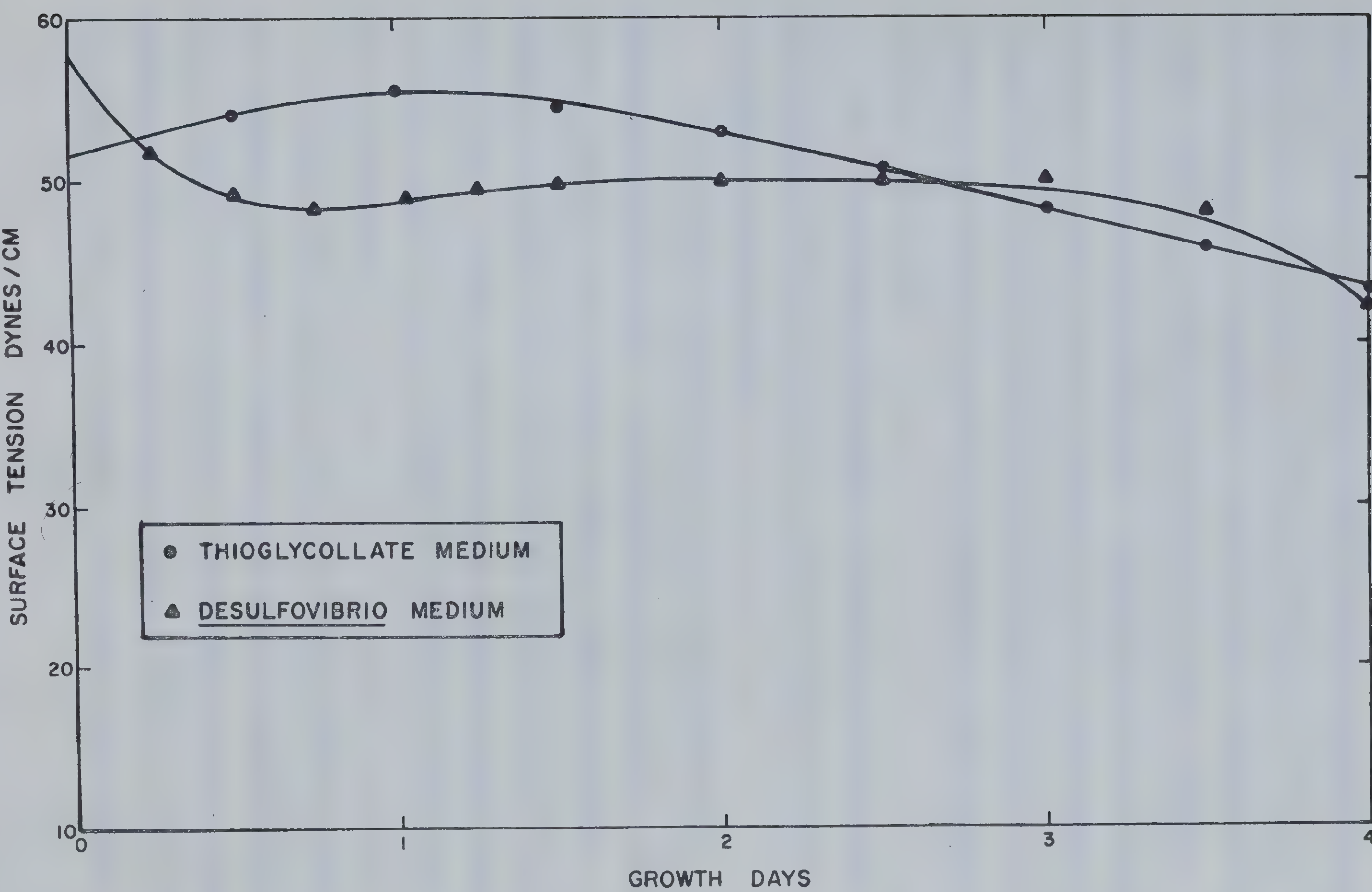

FIG. 9 CHANGE IN SURFACE TENSION OF THIOGLYCOLLATE AND <u>DESULFOVIBRIO</u> MEDIA DURING THE BACTERIAL OIL RELEASE FROM ATHABASCA OIL SAND

La Rivière (120, 121) studied the surface tension lowering effect of bacterial activity as the most important mechanism for oil release by microorganisms. He showed that the general effect of microbial growth is to gradually reduce the surface tension of the culture medium. La Rivière, while showing the general effect of microbial growth upon the lowering of surface tension, was primarily interested in sulfate-reducing bacteria as an oil release agent. He determined that the reduction in surface tension values was due, in part, to autolysis of the cells in *Desulfovibrio desulfricans* cultures 3-4 weeks old.

No sulfate-reducing organisms were detected in this work. *Desulfovibrio* medium was used only to test the probable presence of sulfate-reducing bacteria.

In this work, bacterial oil release using vigorous gas producing organisms seems to be independent of the surface tension effects upon the culture medium. However, without knowing the changes in the other interfacial tensions (solid-water, solid-air, and oil-water, oil-air), one cannot state categorically the effect of surface tension in the attachment of gas bubbles to both solid and oil particles.

Since carbon dioxide is only very slightly soluble in water at atmospheric pressure, it bubbles up through the nutrient medium and is released into the atmosphere. Tiny bubbles of carbon dioxide, produced by respiring bacteria, have a buoyant effect on oil droplets. Very small oil flecks are conveyed to the surface of the nutrient

medium by attached gas bubbles. Some of the released oil remains in a film, floating on the surface of the nutrient medium in which bacteria are growing.

Bowman (122) reported that in the hot water separation method, high speed photographs show the conveyance of bitumen particles to the water surface by attached bubbles. Analysis of the bubbles indicates that they are largely air and water vapor, not hydrocarbon gases. It is believed that this air attachment occurs during the pulping step when there is sufficient mechanical energy to overcome the similar electrical charges on both the bitumen particles and air bubbles. The attachment of air bubbles is preceeded by adsorption of surfactants at the bubble surface and then followed by complete attachment of bitumen around the air bubble.

In this investigation similar results were observed by comparing the bacterial oil release to the results found using water with dissolved carbon dioxide at 30°C. The surface tension of the liquid was 61.20 dynes/cm at 22°C. The results found in this work seem to indicate that the attachment of bitumen to carbon dioxide bubbles in both bacterial oil release and carbonated water oil release from the sands follows a mechanism similar to that of bitumen attachment to air bubbles which has been reported in the hot water separation process.

The above is not to underestimate the role of attachment of bacteria to solid surfaces and/or the production of wetting agents. Many bacteria attach tenaciously to solid surfaces. There is little doubt that the electric charge on the bacterial cell surfaces influences their adherence to solid surfaces. It may be experimentally verified that bacteria adhere more readily to positively charged

surfaces than to negatively charged ones and that bacteria adhere more readily to hydrophilic surfaces than to hydrophobic (123). Secreted metabolic products could also be a factor concerning bacterial attachment to solid surfaces.

"The bulk of the Athabasca tar sands, on the order of 99% is not in direct contact with the bitumen but is 'protected' by an envelope of water. This film of water is further encased by bitumen also partially filling the voids between the individual sand grains. A proportion of mineral matter is in the form of clay and other materials of minus 44-micron size" (124). This film of water could attract the negatively charged bacterial cell surfaces. ZoBell (125) suggested that the affinity of bacteria to solid surfaces promotes the removal of films of oil from oil-bearing materials.

He also stated (126) that different physiological types of bacteria play an important role in the separation of oil from solid sediments. He showed that sulfate-reducing bacteria (Desulfovibrio species) are effective organisms in the bacterial oil release process.

Davies (127) pointed out that sulfate-reducing bacteria have at least three characteristics that dictate against their use in petroleum reservoirs for the release of oil from reservoir rocks. These include their corrosive effects with respect to iron and steel, the plugging effects of colloidal iron sulfide produced by these bacteria, and finally the relatively slow metabolic activity of sulfate-reducing bacteria, particularly regarding utilization of hydrocarbons.

Microscopic and cultural characteristics of the naturally occuring organisms in the Athabasca tar sands found in this investigation show that the isolates do not belong to the Desulfovibrio genus (128). Taking into consideration the gas producing, motility and facultative anaerobic characteristics of the naturally occuring organisms in the Athabasca oil sand, there are promising indications of possible field applications of the bacterial oil release process using the microorganisms which have been found in this investigation.

CONCLUSIONS

Based on the microorganisms tested, and within the limitations of the laboratory procedures utilized, the following conclusions can be drawn:

1. It is evident from the studies presented here, together with those of other investigators, that under favorable conditions microorganisms can be found which are capable of attacking practically any hydrocarbon. However, the heavier oils become more difficult to attack as the viscosity and molecular weight increase. This is due in part to the fact that the more viscous oils are harder to disperse in a liquid medium and hence there is less surface exposed to the growth of microorganisms. But the difficulty of attack is probably also attributable to the larger molecules.

2. Slight oil specific gravity and viscosity reductions were obtained in the presence of *Clostridium sporogenes* cultures. Reduced pressure distillation showed an increase of the intermediate fractions after the treatment. Carbon dioxide and methane seem to be the principal gaseous products resulting from anaerobic respiration of the organisms. The carbon dioxide presence could be very significant in lowering the oil viscosity at reservoir conditions of pressure and temperature.

3. Considerable oil viscosity reductions were obtained with *Clostridium roseum* cultures, probably the result of unstable chemical emulsions. The effects on the amount of intermediate fractions and the gaseous metabolic products were similar to those obtained with *C. sporogenes*.

4. In addition to carbon dioxide and methane, ethane was produced when growing Clostridium sporogenes and Clostridium roseum in a Thioglycollate medium with a Lloydminster crude oil substrate.

5. The Lloydminster reservoir water showed a very high mineralization and does not seem suitable for the growth of microorganisms.

6. Microbial oxidations of the Lloydminster crude oil by single and mixed aerobic organisms resulted in an increase in oil specific gravity and viscosity. Reduced pressure distillations showed an increase in the proportions of the heavy fractions.

7. Similar results were obtained after treatment with enrichment cultures isolated from soils sampled near the Lloydminster refinery.

8. Permanent emulsions were obtained with Mycobacterium rhodochrous. The viscosities of these emulsions were observed to be several times that of the original oil.

9. Oil release from the Athabasca oil sand was obtained by employing naturally occuring bacteria in the sands. Microscopic and cultural characteristics of the organisms showed that the isolates were different from the Desulfovibrio species.

10. The mechanism of the bacterial oil release appears to be the conveyance of bitumen particles to the nutrient medium surface by attached carbon dioxide (metabolic product) bubbles. It is also possible that the bacterial affinity for solid surfaces promotes the removal of oil from oil-bearing sediments.

RECOMMENDATIONS

The modification of natural hydrocarbons by microbial action was investigated in this study. Additional investigation should be made to determine if other microorganisms can be found which will further improve heavy crude oil recovery. Further studies of the naturally occuring organisms in the Athabasca oil sand found in this investigation should be conducted so as to determine possible field applications.

Although the mechanism of microbial modification of hydrocarbons is still under investigation, there are enough reference sources to determine the action of certain microorganisms upon specific hydrocarbons. An analysis of the Lloydminster crude oil, especially of the heavy components, should be made in order to select and test the most suitable organisms.

The indirect modifications of hydrocarbons by metabolic products of the organisms, as well as the microbial modifications of the heavy crude oil via oxidation have been studied in this work. This should be followed by an investigation of chemical reduction effects using organisms that produce hydrogen dehydrogenases.

Additional investigation should be devoted to the determining of the liquid metabolic products of *Clostridium roseum* cultures in the presence of heavy crude oil. It could be the key to the selection of suitable chemical products for reducing oil viscosity.

It is recommended that the nutrient requirements for mixed cultures be studied. In this regard emphasis should be placed on tests using the Lloydminster crude oil as the only source of carbon

since the presence of appreciable amounts of organic materials in the medium discourages hydrocarbon utilization.

Gaseous products (carbon dioxide and methane) of anaerobic organisms should be quantitatively measured to determine their actual importance in eventual field applications.

Taking into consideration the gas producing, motility and facultive anaerobic characteristics of the organisms described in this work, there are promising indications of possible field applications to obtain oil release from the oil sands by "in situ" methods if corrosion problems are not encountered and if the bacterial population effects on the permeability are not very detrimental. Additional research is needed in these areas.

Another possibility is to use the organisms found in this investigation instead of the separation process wherein hot water, under conditions of agitation and access to air, is employed. Since the bacterial oil release process does not involve heating and agitation facilities, the heat and power requirements could be reduced. Further investigation in this field is also recommended.

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APPENDIX A

UTILIZATION OF HYDROCARBONS BY MICROORGANISMS

TABLE A-1

UTILIZATION OF HYDROCARBONS BY MICROORGANISMS

Hydrocarbons	Data of Fühs	Data of Van der Linden and Thijsee
Methane	*Methanobacterium methanooxidans*	*Bacillus methanicus* or *Methanomonas methanica* or *Pseudomonas methanica*
	Mycobacterium flavum var. *methanicum*	*Mycobacterium*
C_2-C_5 n-Paraffins	*Methanobacterium methanooxidans*	*Mycobacterium paraffinicum* *Pseudomonas methanica*
	Bacterium hidium	*Mycobacteria*
	Pseudomonas propanica	
	Pseudomonas sp.	
	Bacterium aliphaticum	
	Bacterium aliphaticum liquefaciens	
	Pseudomonas aeruginosa and *Pseudomonas desmolytica*	
	Pseudomonas aeruginosa Group 473	
	Achromobacter sp.	
	Bacillus. Groups 1, 2	
	Mycobacterium flavum var. *methanica*	
	Mycobacterium methanicum	
	Mycobacterium perrugosum var. *ethanicum*	
	Mycobacterium sp.	
	Mycobacterium paraffinicum	
	Nocardia sp.	

TABLE A-1 (continued)

Hydrocarbons	Data of Fühs	Data of van der Linden and Thijsee
	Acremonium sp.	
C_6-C_{12} n-Paraffins	*Methanobacterium methanooxidans*	*Pseudomonas aeruginosa*
	Bacterium aliphaticum	*Micrococcus*
	Bacterium aliphaticum liquifaciens	*Nocardia petroleophila*
	Pseudomonas fluorescens	*Actinomycete* (*Nocardia hydrocarbonoxydans*)
	Pseudomonas fluorescens and *P. putida*	*Pseudomonas pyocyanea*
	Pseudomonas aeruginosa	
	Pseudomonas aeruginosa and *P. desmolytica*	
	Achromobacter sp.	
	Achromobacter sp. and *Alcaligenes sp.*	
	Micrococcus sphaeroides	
	Micrococcus (*paraffinicus?*)	
	Bacillus. Groups 1,2	
	Corynebacterium sp.	
	Mycobacterium flavum var. *methanica*	
	Mycobacterium perrugosum var. *ethanica*	
	Mycobacterium rubrum var. *propanica*	
	Mycobacterium phlei, *Mycobacterium smegmatis*	
	Nocardia petroleophila	

TABLE A-1 (continued)

Hydrocarbons	Data of Fűhs	Data of Van der Linden and Thijsee
	Nocardia opaca	
	Candida lipolytica	
C_{12}-C_{20} n-Paraffins	*Bacterium aliphaticum*	*Micrococcus cerificans*
	Bacterium aliphaticum liquifaciens	*Pseudomonas*
	Pseudomonas aeruginosa	*Corynebacterium*
	Pseudomonas fluorescens (probable)	
	Achromobacter sp., *Alcaligenes*	
	Microccus (*paraffinicus?*)	
	Paraffinbacterium	
	Cornyebacterium sp. (*Arthrobacter CONN*)	
	Mycobacterium	
	Nocardia petroleophila	
	Nocardia opaca	
	Candida lipolytica	
	Aspergillus versicolor	
C_{20}-C_{34} n-Paraffins	*Bacterium aliphaticum*	
	Bacterium aliphaticum liquifaciens	
	Pseudomonas aeruginosa	
	Paraffinbacterium	
	Corynebacterium sp.	
	Nocardia sp.	
	Candida lipolytica	
	Aspergillus versicolor	

TABLE A-1 (continued)

Hydrocarbons	Data of Fühs	Data of Van der Linden and Thijsse
Isoparaffins	*Bacterium aliphaticum*	*Pseudomonas aeruginosa*
	Pseudomonas fluorescens (probable)	
Phenylalkanes and Cycloalkylalcanes		*Nocardia opaca* (*Proactinomyces opacus*)
		Nocardia salmonicolor
		Mycobacterium
		Pseudomonas
Olefins	*Methanobacterium*	*Candida lipolytica*
	Pseudomonas fluorescens and *P. putida*	*Pseudomonas sp.*
	Bacterium aliphaticum	*Nocardia sp.*
	Bacterium aliphaticum liquefaciens	*Candida lipolytica*
	Pseudomonas aeruginosa	
	Paraffinbacterium	
	Pseudomonas fluorescens	
	Corynebacterium sp.	
	Mycobacterium sp.	
	Mycobacterium lacticola	
	Mycobacterium paraffinicum	
	Mycobacterium phlei	
	Mycobacterium testudo	
	Candida lipolytica	

TABLE A-1 (continued)

Hydrocarbons	Data of Fühs	Data of Van der Linden and Thijsse
Cycloparaffins	Pseudomonas aeruginosa	Pseudomonas aeruginosa
	Pseudomonas fluorescens (probable)	Flavobacterium (unidentified bacterial strain)
	Similar to Micococcus (Rhodococcus) cinnabareus	
	Similar to Micococcus (Rhodoc.) rhodochrous	
Aromatics: Benzene	Pseudomonas aeruginosa	Pseudomonas aeruginosa
		Mycobacterium rhodochrous
		Micrococcus sphaeroides
		Nocardia sp.
		Vibrio sp.
		Micrococcus urea
		Pseudomonas fluorescens
Toluene		Pseudomonas aeruginosa
Naphthalene	Pseudomonas aeruginosa	Pseudomonas aeruginosa
	Pseudomonas scissa, P. pelliculosa, P. pictorum	Nocardia sp.
	Similar to Pseudomonas desmolytica	Bacillus naphthalinicum non-liquefaciens
	Similar to Pseudomonas rathonis	
	Pseudomonas boreopolis	
	Pseudomonas sp.	
	Pseudomonas salopia (Achromobacter salopium)	

TABLE A-1 (continued)

Hydrocarbons	Data of Fühs	Data of Van der Linden and Thijsee
	Pseudomonas arvilla (*Achromobacter arvillum*)	
	Bacterium naphthalinicum non-liquifaciens	
	Vibrio neocistes	
	Vibrio cuneata	
	Achromobacter sp.	
	Flavobacterium sp.	
	Bacterium naphthalinicum	
	Micrococcus sphaeroides	
	Mycobacterium spec. (*Orangegelbes pigment*)	
	Nocardia sp.	
	Nocardia opaca	
	Mycobacterium convolutum (similar to *Nocardia opaca*)	
	Nocardia actinomorpha	
Methyl naphthalene		*Pseudomonas desmolyticum*
		Pseudomonas aeruginosa
Ethylbenzene and Alkylbenzene	*Pseudomonas aeruginosa*	*Nocardia sp*.
	Achromobacter spec.	*Aspergillus niger*
	Micrococcus sphaeroides	*Nocardia opaca*
	Mycococcus rhodochrous (*Mycobacterium rhodochrous*)	
	Mycobacterium spec. (*Orangegelbes pigment*)	
	Nocardia corallina	
	Nocardia sp.	

TABLE A-1 (continued)

Hydrocarbons	Data of Führs	Data of Van der Linden and Thijsee
Anthracene	*Pseudomonas aeruginosa*	*Flavobacterium*
	Flavobacterium spec.	*Pseudomonas aeruginosa*
	Bacterium toluolicum	
	Micrococcus sphaeroides	
	Mycobacterium spec. (orangegelbes pigment)	
	Nocardia spec.	
Phenanthrene and methyl-phenentrene	*Pseudomonas aeruginosa*	*Flavobacterium sp.*
	Pseudomonas boreopolis	*Pseudomonas aeruginosa*
	Achromobacter spec.	
	Bacillus phenanthreni-cum bakiensis	
	Bacillus phenanthreni-cum	
	Bacterium toluolicum	
	Mycobacterium spec.	
	Nocardia sp.	

APPENDIX B

RESPIRATION OF MICROORGANISMS

TABLE B-1

MICROBIAL RESPIRATION BY OXIDATION OF INORGANIC SUBSTANCES

Organism	Reaction
Hydrogenomonas	$6H_2 + 2O_2 + CO_2 \rightarrow 5H_2O + \underset{\text{cell material}}{(CH_2O)}$
Beggiatoa	$2H_2S + CO_2 \rightarrow \underset{\text{stored in cells}}{2S} + \underset{\text{cell material}}{(CH_2O)} + H_2O$ In absence of H_2S: $2S + 5H_2O + 3CO_2 \rightarrow 2SO_2^{=} + \underset{\text{cell material}}{3(CH_2O)} + 4H^+$
Thiobacillus	$S_2O_3^{=} + H_2O + 2O_2 \rightarrow 2SO_4^{=} + 2H_2^+$
Nitrosomonas	$2NH_3 + 3O_2 \rightarrow 2NO_2^- + 2H^+ + 2H_2O$
Nitrobacter	$2NO_2^- + O_2 \rightarrow 2NO_3^-$

TABLE B-2

ANAEROBIC RESPIRATIONS

Organism	Reaction
Many species	$NO_3 \xrightarrow{H} NO_2 \xrightarrow{H} N_2\uparrow$; $NO_2 \xrightarrow{H} NH_4^+$
	"Sulfate reduction"
<u>Desulfovibrio</u>	$CH_3COOH + SO_4^{=} \rightarrow 2CO_2 + H_2S + 2OH^-$
	"Methane fermentation"
Various species	Organic compounds $\xrightarrow{\text{anaerobic}} CO_2 + CH_4 + \underset{\text{cell material}}{(CH_2O)}$
<u>Methanobacterium omelianskii</u>	$2C_2H_5OH + CO_2 \rightarrow 2CH_3COOH + CH_4$
Various species	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$
Various species	$CH_3COOH \rightarrow CH_4 + CO_2$
<u>Clostridium aceticum</u>	$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$

APPENDIX C

RESERVOIR FLUID PROPERTIES

TABLE C-1

DETERMINATION OF THE THIXOTROPIC CHARACTERISTICS OF THE ORIGINAL CRUDE OIL

Rate of Shear	Shearing Stress
0.3	0.7
0.6	1.8
1.5	4.4
3.0	8.9
6.0	17.9
12.0	35.9
30.0	89.2
12.0	33.4
6.0	18.0
3.0	9.5
1.5	5.1
0.6	2.8
0.3	2.0

Note: Measurements with the Brookfield Rotating Viscometer, Model LVT. All the measurements were taken with the Spindle #3, after five minutes of agitation.

TABLE C-2

VISCOSITY-TEMPERATURE RELATIONSHIP OF THE ORIGINAL CRUDE OIL AT ATMOSPHERIC PRESSURE

Temperature °C	Oil viscosity, measured with the #3 spindle at 30 rpm, after 60 sec of agitation cps
6.00	24,850
13.00	11,240
15.00	8,500
20.00	4,050
23.00	3,016
25.50	2,100
30.00	1,460
33.50	1,148
37.00	1,016

TABLE C-3

ANALYSIS OF RESERVOIR WATER

Appearance of Sample: Clear and colorless solution with red sediment on the bottom.

	Mg per liter
Cl	47,646
OH	0
HCO_3	74
CO_3	0
SO_4	18
Ca	3,175
Mg	936
Na (calc)	25,525
Total Solids (calc)	77,374

Specific gravity, 60°F	1.0495
pH	6.95
H_2S	None
Resistivity, ohm-meters, 25°C	.101
Refractive index, 25°C	1.3456
Total solids evap. at 110°C	128,530

Remarks: Fe in original solution

APPENDIX D

OIL PHYSICAL PROPERTIES AFTER MICROBIAL TREATMENT

TABLE D-1

EFFECTS ON THE PHYSICAL PROPERTIES OF THE LLOYDMINSTER CRUDE OIL CAUSED BY METABOLIC PRODUCTS OF ANAEROBIC MICROORGANISMS

Organism	Nutrient Medium	Gas Production	Oil Specific Gravity at 60°F	Oil Viscosity at 23°C, measured with the #3 spindle at 30 rpm, after 60 sec. of agitation cps
Original crude oil (control)			.9734	3,016
Cl. sporogenes	Thioglycollate	+	.9730	1,850
Cl. roseum	Thioglycollate	+	.9730	400
Cl. septicum	Thioglycollate	+	.9745	3,200
Cl. rubrum ATCC 14949	Thioglycollate	+	.9732	3,000
Cl. rubrum ATCC 14950	Thioglycollate	+	.9734	3,140
Cl. butylicum	Thioglycollate	+	.9740	4,200
Cl. acetobutylicum	Thioglycollate	+	.9738	3,500
Cl. thermosaccharolyticum	Molasses 2%	+	.9745	4,600
Cl. roseum	Molasses 2%	+	.9730	420
D. desulfricans and *Cl. sporogenes*	*D. desulfricans* medium	+	.9740	4,320

TABLE D-2

EFFECTS ON THE PHYSICAL PROPERTIES OF THE LLOYDMINSTER CRUDE OIL CAUSED BY AEROBIC ORGANISMS

Organism	Nutrient Medium	Oil Specific Gravity at 60°F	Oil Viscosity at 23°C measured with the #3 spindle at 30 rpm, after 60 sec. of agitation cps	Incubation temperature °C	Incubation time days
Original crude oil		.9734	3,016		
Crude oil, after 10 days agitation in water (control)		.9734	3,490		
Crude oil, after 30 days agitation in water (control)		.9735	3,620		
P. aeruginosa	M. salts (1)	.9742	3,720	37	10
P. fluorescens	M. salts (1)	.9750	3,800	26	10
P. rubescens	M. salts (1)	.9738	3,660	26	10
P. oleovorans	M. salts (1)	.9840	4,080	26	10

TABLE D-2 (continued)

Organism	Nutrient	Oil Specific Gravity at 60°F	Oil Viscosity at 23°C measured with the #3 spindle at 30 rpm, after 60 sec. of agitation cps	Incubation temperature °C	Incubation time days
Mycobacterium rhodochrous	M. salts (2)	.9772	6,460	30	4
Mycobacterium rhodochrous	M. salts	.9800	16,080	30	10
Scopulariopsis koningii	M. salts (3)	.9750	5,280	26	10
C. lipolytica	Tripticase	.9740	5,140	26	10
C. tropicalis	Tripticase	.9742	5,320	26	10
Penicillium roqueforti	Tripticase	.9770	5,080	26	10
Mixed culture #1	Nutrient Agar Difco	.9860	6,440	26	30
Mixed culture #2	Nutrient Agar Difco	.9796	5,260	26	30

TABLE D-2 (continued)

Organism	Nutrient	Oil Specific Gravity at 60°F	Oil Viscosity at 23°C measured with the #3 spindle at 30 rpm, after 60 sec. of agitation cps	Incubation temperature °C	Incubation time days
Mixed culture #3	Nutrient Agar Difco	.9800	5,350	26	30
Mixed culture #3	M. salts (4)	.9840	5,600	26	30
Mixed culture #4	Nutrient Agar Difco	.9780	4,900	37	30

Media Composition, g per liter distilled water

(1) K_2HPO_4, 0.5; $MgSO_4$, 0.5; Na_2HPO_4, 1.0; NH_4Cl, 0.5; NaCl, 4.0

(2) NH_4Cl, 0.5; $(NH_4)_2SO_4$, 0.05; NaCl, 0.4; $MgCl_2$, 0.1; Na_2HPO_4, 6.0; KH_2PO_4, 3.0

(3) $MgSO_4$, 0.5; Na_2HPO_4, 1.0; KH_2PO_4, 0.5; NH_4NO_3, 2.5; $CaCO_3$, 5.0

(4) $MgSO_4$, 0.2; Na_2HPO_4, 1.0; KH_2PO_4, 1.0; NH_4NO_3, 1.0; NaCl, 0.5; $CaCl_2$ trace; $CaCO_3$, 5.0; $FeCl_3$ trace.

APPENDIX E

EFFECT OF MOLASSES CONCENTRATION AND THE PRESENCE OF HYDROCARBONS ON THE GROWTH OF *CLOSTRIDIUM*

TABLE E-1

EFFECT OF MOLASSES CONCENTRATION ON THE GROWTH OF CLOSTRIDIUM

Organism	Molasses 1%	2%	3%	4%	5%
Cl. roseum	+	+++	++	++	+
Cl. rubrum	++	+++	+++	++	+
Cl. septicum	-	+	+	-	-
Cl. sporogenes	+++	+++	+++	++	++
Cl. thermosaccharolyticum	+++	+++	++	++	+

Note:

+++ Optimal Growth

++ Good growth

+ Growth

- No Growth

APPENDIX F

REDUCED PRESSURE DISTILLATIONS

TABLE F-1

REDUCED PRESSURE DISTILLATION OF THE ORIGINAL CRUDE OIL

Per cent Recovered %	Temperature °C	Pressure mm Hg
I.B.P.	30.00	4
2.00	52.00	4
2.50	106.00	4
5.00	118.00	4
7.50	133.00	4
10.00	145.00	4
12.50	155.00	4
15.00	166.00	4
17.50	177.00	4
20.00	189.00	4
22.50	201.00	4
25.00	214.50	4
27.50	227.00	4
30.00	238.00	4
F.B.P.	241.00	4

Recovery, per cent.... 32.00

Residue, per cent.... 68.00

TABLE F-2

REDUCED PRESSURE DISTILLATION OF THE OIL AFTER TREATMENT WITH *CLOSTRIDIUM SPOROGENES*

Recovery Per Cent %	Temperature °C	Pressure mm Hg
I.B.P.	30.00	4
2.00	76.00	4
2.50	87.00	4
5.00	115.00	4
7.50	121.50	4
10.00	139.00	4
12.50	150.50	4
15.00	156.00	4
17.50	160.00	4
20.00	163.00	4
22.50	177.00	4
25.00	193.00	4
27.50	204.50	4
30.00	220.50	4
32.50	231.00	4
35.00	239.00	4
F.B.P.	242.00	4

Recovery, per cent.... 35.00

Residue, per cent.... 65.00

TABLE F-3

REDUCED PRESSURE DISTILLATION OF THE OIL AFTER TREATMENT WITH *CLOSTRIDIUM ROSEUM*

Per Cent Recovered %	Temperature °C	Pressure mm Hg
I.B.P.	30.00	4
2.00	78.00	4
2.50	90.00	4
5.00	112.00	4
7.50	120.00	4
10.00	138.00	4
12.50	147.50	4
15.00	152.50	4
17.50	157.00	4
20.00	160.00	4
22.50	176.00	4
25.00	190.00	4
27.50	201.00	4
30.00	218.00	4
32.50	230.00	4
35.00	236.00	4
F.B.P.	241.00	4

Recovery, per cent.... 35.50

Residue, per cent.... 64.50

TABLE F-4

REDUCED PRESSURE DISTILLATION OF THE
OIL AFTER TREATMENT WITH *PSUEDOMONAS AERUGINOSA*

Recovery Per Cent %	Temperature °C	Pressure mm Hg
I.B.P.	37.00	4
2.00	80.00	4
2.50	97.00	4
5.00	120.00	4
7.50	131.00	4
10.00	147.50	4
12.50	153.50	4
15.00	171.00	4
17.50	180.00	4
20.00	197.00	4
22.50	210.00	4
25.00	222.50	4
27.50	233.00	4
30.00	240.00	4
F.B.P.	243.00	4

Recovery, per cent....	30.00
Residue, per cent....	70.00

TABLE F-5

REDUCED PRESSURE DISTILLATION OF THE OIL AFTER TREATMENT WITH MIXED CULTURE #3

Recovery Per Cent %	Temperature °C	Pressure mm Hg
I.B.P.	53.00	4
2.00	87.50	4
2.50	102.00	4
5.00	127.00	4
7.50	139.00	4
10.00	160.00	4
12.50	172.50	4
15.00	183.00	4
17.50	197.00	4
20.00	203.50	4
22.50	220.00	4
25.00	240.00	4
F.B.P.	242.00	4

Recovery, per cent.... 25.00

Residue, per cent.... 75.00

TABLE F-6

REDUCED PRESSURE DISTILLATION OF THE OIL AFTER TREATMENT WITH ENRICHMENT CULTURE

Recovery Per cent %	Temperature °C	Pressure mg Hg
I.B.P.	62.00	4
2.00	85.00	4
2.50	101.50	4
5.00	129.00	4
7.50	142.00	4
10.00	158.50	4
12.50	167.00	4
15.00	176.00	4
17.50	196.00	4
20.00	205.00	4
22.50	227.00	4
25.00	240.00	4
F.B.P.	242.00	4

Recovery, per cent.... 25.50

Residue, per cent.... 74.50

APPENDIX G

GAS CHROMATOGRAPHIC ANALYSIS OF METABOLIC PRODUCTS

TABLE G-1

RESULTS OF CHROMATOGRAPHIC ANALYSIS OF THE GAS PRODUCED BY *CLOSTRIDIUM ROSEUM* WITHOUT CRUDE OIL SUBSTRATE

Component	Weight, per cent
H_2	14.63
N_2	.89
O_2	.16
CO_2	84.31
Total	99.99

TABLE G-2

RESULTS OF CHROMATOGRAPHIC ANALYSIS OF THE GAS PRODUCED BY *CLOSTRIDIUM ROSEUM* WITH CRUDE OIL SUBSTRATE

Component	Weight, per cent
CH_4	.61
C_2H_6	.31
N_2	26.79
O_2	6.58
CO_2	65.73
Total	100.02

TABLE G-3

RESULTS OF CHROMATOGRAPHIC ANALYSIS OF THE GAS PRODUCED BY *CLOSTRIDIUM SPOROGENES* WITH CRUDE OIL SUBSTRATE

Component	Weight, per cent
CH_4	2.57
C_2H_6	.87
N_2	70.76
CO_2	25.81
Total	100.01

TABLE G-4

RESULTS OF CHROMATOGRAPHIC ANALYSIS OF THE GAS PRODUCED BY MIXED CULTURE #3 WITH LLOYDMINSTER CRUDE OIL SUBSTRATE

Component	Weight, per cent
N_2	79.30
O_2	11.54
CO_2	9.16
H_2S	Trace
Total	100.00

TABLE G-5

RESULTS OF CHROMATOGRAPHIC ANALYSIS OF THE GAS PRODUCED BY NATURALLY OCCURRING MICROORGANISMS IN THE ATHABASCA OIL SAND

Component	Weight, per cent
H_2	.64
N_2	47.53
O_2	1.16
CO_2	50.69
H_2O (vapor)	Trace
Total	100.02